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Not Just the Brain: Methamphetamine Disrupts Blood-Spinal Cord Barrier and Induces Acute Glial Activation and Structural Damage of Spinal Cord Cells

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Abstract

Acute methamphetamine (METH) intoxication induces metabolic brain activation as well as multiple physiological and behavioral responses that could result in life-threatening health complications. Previously, we showed that METH (9 mg/kg) used in freely moving rats induces robust leakage of blood-brain barrier (BBB), acute glial activation, vasogenic edema, and structural abnormalities of brain cells. These changes tightly correlated with drug-induced brain hyperthermia and were greatly potentiated when METH was used at warm ambient temperatures (29°C), inducing more robust and prolonged hyperthermia. Extending this line of research, here we show that METH also strongly increases the permeability of the blood-spinal cord barrier (BSCB) as evidenced by entry of Evans blue and albumin immunoreactivity in T9-12 segments of the spinal cord. Similar to the BBB, leakage of BSCB was associated with acute glial activation, alterations of ionic homeostasis, water tissue accumulation (edema), and structural abnormalities of spinal cord cells. Similar to that in the brain, all neurochemical alterations correlated tightly with drug-induced elevations in brain temperature and they were enhanced when the drug was used at 29°C and brain hyperthermia reached pathological levels (>40°C). We discuss common features and differences in neural responses between the brain and spinal cord, two inseparable parts of the central nervous system affected by METH exposure.

Abstract

We show that METH administration disrupts blood-spinal cord barrier to proteins, inducing edema and cell injury that were more pronounced when the drug was administered at 29°C than at 23°C.

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CONFLICT OF INTEREST

The authors report no conflict of interests

Keywords

Albumin leakage; Blood-spinal cord barrier; Brain; Cellular damage; GFAP; hyperthermia; Metabolic brain activation; Spinal cord

INTRODUCTION

Hyperthermia is the most prominent symptom of acute methamphetamine (METH) intoxication and is a factor implicated in neurotoxicity during chronic drug use [1-3]. Recently, we reported that METH induces robust leakage of the blood-brain barrier (BBB), acute glial activation, brain tissue water accumulation (edema), and structural abnormalities of brain cells [4, 5]. Although the extent of these changes differed in the cortex, thalamus, hypothalamus, and hippocampus, in each structure they were tightly related to drug-induced brain temperature elevation. When the rats were exposed to METH at a warm (29°) ambient temperature, they showed much stronger brain and body hyperthermic response as well as larger shifts in histochemical and morphological parameters [6].

While the brain is the predominant substrate in studies related to physiological and pathophysiological effects of addictive drugs, in the present work, we focused on the spinal cord, another important, but often less appreciated part of the CNS. Similar to the BBB that reliably protects the brain's neurochemical homeostasis from potentially dangerous influences arising from the peripheral blood, spinal cord tissue is equipped with blood-spinal cord barrier (BSCB) that have similar structure and functions [7-11]. Although the data on BSCS are more limited than for BBB, dysfunctions of this barrier could occur during spinal cord injury [12-16], damage or inflammation of spinal nerves [17, 18], and different toxic or neurotoxic insults [7, 19], thus contributing to the various symptoms associated with these conditions (i.e., edema, chronic neuropathic pain, and etc.)

The present study is an extension of our previous work aimed at the study of neurochemical and structural alterations induced by acute METH intoxication and their underlying mechanisms. While our previous work was focused on the brain [4, 5], here we examined whether similar changes in barrier functions, glial activity and cellular morphology occur in the spinal cord. Several goals were formulated in this study. First, by evaluating Evans blue entry and albumin immunoreactivity in spinal cord tissue, we determined whether acute METH exposure (9 mg/kg) results in leakage of BSCB, how this leakage depends on brain and body temperature, and how it differs from a similar process occurring in the brain. Second, by examining changes in ionic homeostasis (Na⁺, K⁺, Cl⁻) and water content in spinal cord tissue, we assessed whether acute METH intoxication results in edema and how it differs from edema seen in brain structures. Third, since acute METH intoxication results

in rapid glial activation evident to a different extent in all tested brain structures, we evaluated METH-induced changes in immunoreactivity for glial fibrillary acidic protein (GFAP), an index of astrocytic activation [18, 20]. Finally, we examined to what extent spinal cord cells are prone to structural damage that occurs selectively in different brain structures during acute METH exposure. To further explore the role of hyperthermia as a critical factor affecting METH-induced functional and structural changes, in addition to the animal group exposed to METH at standard laboratory conditions, the same parameters were also examined in rats exposed to METH at the same dose but at warm (29°C) ambient temperature, when rats developed extreme brain and body hyperthermia (>40°C). Since multiple parameters were obtained for the spinal cord and brain structures in the same animals, we were able to evaluate quantitative relationships between drug-induced brain temperature elevation, permeability of the BSCB, and morphological abnormalities of spinal cord cells as well as to assess the common features and differences between the spinal cord and brain. To our knowledge, this is the first study, which provides a detailed characterization of acute drug-induced BSCB alterations, glial activation, and associated structural cell damage in the spinal cord, the first study clarifying these changes for METH, a widely used drug of abuse with neurotoxic potential, and the first study comparing changes between the brain and spinal cord, two inseparable parts of the CNS.

MATERIALS AND METHODS

Animals and Surgery

Data reported in this study were obtained from 20 male Long-Evans rats (420±40 g) supplied by Charles River Laboratories (Greensboro, NC). All animals were housed individually under standard laboratory conditions (12-hr light cycle beginning at 07:00) with free access to food and water. Protocols were performed in compliance with the Guide for the Care and Use of Laboratory Animals (NIH, Publication 865-23) and were approved by the NIDA-IRP Animal Care and Use Committee. Maximal care was taken to minimize both the number of animals used and their possible suffering.

As previously described in detail [21], all animals were implanted with three miniature thermocouple sensors. The tip of the first sensor was located in the nucleus accumbens (NAcc) shell [1.2 mm anterior to bregma, 0.9 mm lateral to bregma, 7.2-7.4 mm below skull surface; according to coordinates of Paxinos and Watson [22], a deep, ventrally located structure that represented brain temperature. To assess skin temperature, the second thermocouple probe was implanted subcutaneously along the nasal ridge with the tip approximately 15 mm from bregma. A third thermocouple probe was implanted in deep temporal muscle (*musculus temporalis*), a non-motor head muscle, which is supplied through the carotid artery by the same arterial blood supply as the brain. The latter location provided a measure of body temperature and, by comparing it with brain temperature, it also served to evaluate the source of brain hyperthermia and its underlying mechanisms [23]. All three probes were secured with dental cement to three stainless steel screws threaded into the skull. During the same surgery, each animal was also implanted with a jugular intravenous (iv) catheter, which run subcutaneously to the head mount and secured with

dental cement. Rats were allowed three days recovery and two days of habituation (6-8 hrs) before the start of testing.

Experimental Protocol

All recordings occurred inside a Plexiglas chamber (32×32m×32 cm) equipped with four infrared motion detectors (Med Associates, Burlington, VT, USA), placed inside of a light- and sound-attenuating chamber. Rats were brought to the testing chamber and attached via a flexible cord and electrical commutator to thermal recording hardware (Thermes 16, Physitemp, Clifton, NJ, USA). A catheter extension was also attached to the internal catheter, thereby allowing remote, stress- and cue-free iv injections. The catheter was filled with 2% solution of Evans blue (EB; Sigma, USA; dissolved in saline), which was injected at a specified time following drug or saline injections (see below). Temperatures were recorded with a time resolution of 10 s and movement was recorded as the number of infrared beam breaks per 1 min.

All animals were divided into three groups (control-23°C, METH-23°C, METH-29°C). After 3 hrs habituation to the testing chamber, control rats (n=4) received a single subcutaneous (sc) saline injection (0.3 ml), while each rat of the other two groups (n=8 each) received a single sc injection of METH (9 mg/kg in 0.3 ml saline) either at 23 or 29°C ambient temperatures. Although 29°C is ~6°C larger than standard laboratory temperatures, it corresponds to temperature neutrality or temperature comfort in rats, when basal metabolism is at its lowest levels [24]. At a specified time following drug or saline injection, each rat was slowly injected with a solution of EB (3 ml/kg over 60 s), 5 min later anesthetized with iv Equithesin (0.8 ml over 30 s), and taken for brain perfusion. In control group, dye was injected 120 min after sc saline injection. In the METH groups, dye was injected at peak brain temperature values or when NAcc temperature exceeded 41.5°C, suggesting a high probability of future lethality (23°C: range 66-94 min, mean 82 min; 29°C: range 26-79, mean 58 min). Animals were perfused with cold 4% paraformaldehyde solution containing 0.5 % glutaraldehyde and 2.5 % picric acid in phosphate-buffered saline (PBS, 0.1 M, pH 7.4) at the rate of 20 ml/min for 10 min. The intravascular blood was washed-out before fixation by 0.1 M PBS (20 ml/min for 5 min). The perfusion pressure was maintained at 100 torr during these procedures. The animals were wrapped in aluminum foil and kept in a refrigerator at 4°C overnight. The next day, the brains and spinal cord were removed and kept in the same fixative at 4°C until processing for further analyses.

Histochemical and morphological measurements and observations

The integrity of the BSCB was evaluated based on leakage of EB on the dorsal and ventral surfaces of the spinal cord, concentrations of EB extracted biochemically from selected loci of spinal cord and measured using spectrophotometric analysis [25], and quantitative immunohistochemistry for albumin conducted in spinal cord slices. All measurements and evaluations were conducted separately in four levels of the spinal cord (T8-T12) [see 9, 18, 26 for details].

Immunohistochemistry for albumin was performed on Vibratome (60 µm thick) sections using a sheep polyclonal anti-rat albumin antibody (Sigma, USA) and the streptavidin-HRP-

biotin technique as described previously [9, 27]. Briefly, the endogenous peroxidase activity was blocked with 3% H₂O₂ and 5% normal goat serum, followed by incubation with the primary antibodies (1:500 for albumin). This was followed by incubation with biotinylated antibody and HRP (Dako), with brief rinses in PBS between incubations. The reaction was visualized using 3-amino-9-ethycarbazole (Vector Laboratories, Burlingame, USA) and counterstained with hematoxylin. Reagent controls (omitting the primary antibody or substituting non-immune serum for the primary antibody in the staining protocol) on tissue sections were used to confirm the specificity of the primary antibodies used [for details see 18, 20]. The numbers of albumin-positive cells (irrespective of their presence in neurons or glial cells) was counted in one half of the brain slices from each animal, in a blinded fashion three times. The median value was used for final calculation [5].

5- μ m paraffin sections of spinal cord were used for quantitative evaluation of GFAP immunoreactivity, conducted by using a commercial protocol. In brief, after deparaffinization, endogenous peroxidase was inhibited with 0.3 % hydrogen peroxide with 1 % non-immune horse serum in phosphate buffered saline (PBS, pH 7.4) for 20 min and then for 8 h with monoclonal anti-GFAP serum (DAKO, Hamburg, Germany) diluted 1:500 in PBS. After incubation with biotinylated horse anti-mouse immunoglobulin IgG at a 1:50 dilution and avidin-biotin complex (ABC) (Vector Laboratories, Burlingame, USA) for 45 min, the brown reaction product was developed with 3,3'-tetraaminobenzidine and hydrogen peroxide in 0.05 M Tris-HCl buffer (pH 7.4) for 4 min [28, 29]. The paraffin sections of the control and METH-treated groups were processed simultaneously in parallel groups. Control sections (counterstained with Haematoxylin-Eosin or incubated in pre-immune horse serum) showed no immunoreaction product. The GFAP immunoreactivity in selected areas of spinal cord was assessed in a blind fashion by at two independent observers. The slides were examined by microscope and photographed. For quantitative analyses, the number of GFAP-positive cells was counted in a blinded fashion. This analysis was done in similar levels of spinal cord (T8-T12) as all other measurements and observations.

The water tissue content was calculated from the differences between dry and wet weights of the sample [30] and ion content (Na^+ , K^+ and Cl^-) was measured from the dry weight of the samples as described earlier [10, 31]. These measurements were made in slices from another half of the spinal cord at the same levels (T8-T12).

Three- μm paraffin sections passing through spinal cord at T8-T12 levels were cut and stained with Haematoxylin and Eosin or Nissl for light microscopy histological analyses of cellular changes. The numbers of damaged neurons showing distortion, swelling or shrinking within the neuropil were calculated separately for each level of the spinal cord [see 9, 15, 28].

Ultrastructural analyses

To study myelin damage caused by METH within the spinal cord, we used transmission electron microscopy [26]. For this purpose, small tissue pieces from the desired areas of the spinal cord were post-fixed in Osmium Tetraoxide (OsO₄) and embedded in Plastic (Epon 812) using standard procedures [9]. About 1 μ m thick section were cut and stained with Toluidine blue for examining the areas that should be analyzed at the ultrastructural level.

After trimming the sections for the desired area of the ventral spinal horn, ultrathin sections were cut using diamond knife at LKB Ultramicrotome (Sweden). Serial ultrathin sections were collected on an one hole grid and counter stained with Lead Citrate and Uranyl Acetate before viewing at Phillips 400 Transmission Electron Microscope. The images were collected using a digital camera Gatan (USA) and stored in a Macintosh Computer for further analyses. The images were obtained at 4 to 6000 magnification to study myelin vesiculation [9, 29, 32].

Data Analyses

Temperature and movement data were analyzed with 2-min time bins and presented as both absolute and relative changes with respect to the moment of drug administration. ANOVA with repeated measures, followed by post-hoc Fisher tests, was used for statistical evaluation of drug-induced changes in temperature and movement. Student's t-test was used for comparing between-site differences in temperature and locomotion. Correlative and regression analyses were used to assess the relationships between temperatures and several histochemical and morphological parameters. For evaluating differences in METH response between in the spinal cord and the brain, these original data obtained in the spinal cord were compared to previously reported data on changes in similar parameters occurring in various brain areas [4, 5].

RESULTS

1. Leakage of BSCB during METH intoxication

As shown in **Fig. 1A**, METH (9 mg/kg, sc) administered to quietly resting rats at standard (23°C) and warm (29°C) ambient temperatures induced robust increases in NAcc and temporal muscle temperatures, which were clearly larger in the latter group. At 23°C, mean NAcc and muscle temperatures peaked at $2.31 \pm 0.22^\circ\text{C}$ and $1.99 \pm 0.24^\circ\text{C}$ above baseline and in the METH-29°C group the increase was significantly larger, $4.13 \pm 0.28^\circ\text{C}$ and $3.68 \pm 0.26^\circ\text{C}$, respectively ($p < 0.05$ for both locations). METH-induced NAcc temperature increases widely varied in individual animals (from 1.2 to 3.0°C at 23°C and from 2.6 to 5.1°C at 29°C), and they were independent of basal temperatures before drug administration ($r = 0.259$). However, NAcc and muscle temperatures at their peak levels were closely interrelated (**Fig. 1B**), showing a strong and almost perfect linear correlation ($r = 0.982$, $p < 0.001$). In each condition, NAcc temperatures were larger than those in temporal muscle and the regression line calculated for all animals (bold hatched line in **Fig. 1B**) was parallel to the line of equality (thin hatched line).

Significant between-group differences were found in concentration of EB, a traditional measure of barrier leakage (**Fig. 1C**). While low levels of EB were found in spinal cord of all control animals (mean 0.15 ± 0.01 mg%), the levels were 3-4-fold larger in the METH-23°C (0.564 ± 0.028 mg%, $p < 0.001$) and 6-7-fold larger (0.996 ± 0.03 mg%, $p < 0.001$ vs. control and METH-23°C) in METH-29°C groups. As shown in **Fig. 1D**, accumulation of Evans Blue in neural tissue was tightly related to drug-induced elevation of brain and muscle temperatures; correlation was exceptionally strong ($r = 0.97$ and 0.96 , respectively) and highly linear. As shown in **Fig. 1C**, the extent of METH-induced leakage was very

similar in BSCB and BBB (white bars). Mean values of EB concentration in brain tissue (sum of cortex, thalamus, hypothalamus, and hippocampus) were slightly higher than in the spinal cord, with minimal but significant differences both in control and METH-29°C groups ($p<0.05$).

Figure 2 shows a schematic diagram of leakage and spread of Evans blue within the spinal cord structures found in METH-treated animals at 23° and 29°C as compared to the control group. As evident from this figure, METH administered at 29°C resulted in more widespread extravasation of the Evans blue dye within the spinal cord segments as compared to the drug given at 23°C. Moreover, METH-induced Evans blue extravasation was markedly different in different levels of the spinal cord segments examined. The intensity and spread area of the dye was most prominent in the 29°C group in the dorsal, ventral and lateral horns in the grey matter of the cord as compared to the 23°C group where the dye was largely located within the dorsal and the ventral grey matter of the cord. The intensity of the dye extravasation was also much larger in the METH-29°C group as compared to the METH-23°C group.

Albumin immunoreactivity is another very sensitive index of barrier leakage. Using this parameter, the between-group differences were exceptionally large (**Fig. 1E**). While only sporadic albumin-containing cells were found in control animals (mean 0.50 ± 0.10 cells/slice), their numbers in the spinal cord increased about 20 (9.50 ± 0.70) and 56-fold (28.34 ± 2.51) larger in METH-23°C and METH-29°C groups, respectively. Albumin immunoreactivity was also tightly related to NAcc and muscle temperatures (**Fig. 1F**); coefficient of correlation was smaller ($r=0.89$) and between-group differences more evident. Similar to EB leakage, albumin immunoreactivity was clearly less intense in spinal cord than in brain tissue (**Fig. 1E**), with significant differences between each animal group (at least $p<0.01$ for each group). **Figure 3A** shows representative examples of albumin leakage within the spinal cord tissue after METH administration at low and high ambient temperatures. As seen in the light micrograph, albumin-positive cells were almost not seen in slices obtained from control, saline-injected rats (a) and they were more frequent in the METH-29°C group (c) as compared to the METH-23°C group (b). Also, the albumin leakage is largely confined to the distorted neurons in both groups. Edematous expansion of the cord is also clearly evident, and it is more pronounced in the rats exposed to METH at 29°C ambient temperatures.

2. Acute glial activation in the spinal cord during METH intoxication

Similar to brain tissue, METH induced strong glial activation in the spinal cord, and the number of GFAP-positive cells was significantly larger when drug was used at 29°C (**Fig. 4A**). Compared to control, the averaged number of GFAP-positive cells in spinal cord was 8- and 16-fold larger in MEH-23°C and METH-29°C groups, respectively. The extent of glial activation in the spinal cord was slightly lower than in brain tissue in each condition, with significant differences both in control and METH-29°C group ($p<0.05$). Acute glial activation was tightly related to leakage of BSCB as evidenced by a strong correlation between the number of GFAP-positive cells and both the concentration of Evans Blue (**Fig. 4B**) and albumin immunoreactivity (**Fig. 4C**). Glial activation was also dependent upon the

extent of METH-induced brain temperature elevation. While in normothermic control only sporadic GFAP-positive cells were found in spinal cord tissue, their number grew to 15-30/slice at peak of temperature elevation in METH-23°C group, and peaked at 40-50 cell/slice during extreme hyperthermia in METH-29°C rats (**Fig. 4D**). **Figure 3B** shows representative examples of spinal cord slices demonstrating differences in GFAP immunoreactivity in animals of different groups. As evident with the figure, the intensity and spread of GFAP immunoreactivity is much less in the METH-23°C group (b). In the latter group, upregulation of GFAP immunoreactivity was seen around large microvessels, whereas in the METH-29°C group many microvessels and perineuronal areas also showed intense GFAP immunoreactivity. Also the sponginess and edema are clearly stronger in the 29°C group compared to control (a) and 23°C groups (b).

3. Alterations in ionic and water balance in the spinal cord during METH intoxication

The ability of brain to maintain highly stable ionic and water homeostasis is essential for maintaining normal physiological and behavioral functions and even relatively small changes in ion and water content in brain tissue result in dramatic impairments in brain activity and functions. Tissue water accumulation (vasogenic edema) is a serious, life-threatening condition that could result in organism's death. Previously we found that acute METH intoxication results in significant water accumulation in brain tissue that becomes stronger when the drug is used at 29°C and brain temperature elevation is larger [4]. A similar, but quantitatively stronger change was found in the spinal cord (**Fig. 5A**). With respect to basal values of tissue water, its content in METH-treated rats grew for 1.7% and 3.0% for 23°C and 29°C, respectively. These range of changes greatly exceeded that seen in the brain (**Fig. 5B**). Another prominent difference found in the spinal cord was ~12% less content of water than that in cerebral structures.

Comparison of ionic response seen in the spinal cord and the brain revealed a general similarity in changes of Na⁺, Cl⁻, and K⁺ concentrations (**Fig. 5C and D**). Both Na⁺ and Cl⁻ increased during METH intoxication, and the increase was larger at 29°C. Changes in K⁺ were weaker, but both in spinal cord and the brain concentration of K⁺ significantly increased in METH-29°C group. Basal levels of all ions did not differ between the spinal cord and the brain.

By using regression analysis, we show that water accumulation in spinal cord tissue directly correlates with the leakage of the BSCB (albumin-positive cells; **Fig. 6A**), glial activation (GFAP-positive cells; **Fig. 6B**) and the magnitude of METH-induced brain temperature elevation (**Fig. 6C**). Within the entire animal sample, correlation was strong for each parameter and maximal for NAcc temperature change.

4. Morphological abnormalities of spinal cord cells resulting from acute METH intoxication

As shown previously, acute METH intoxication results in structural changes of brain cells in all tested brain structures; the number of abnormal cells in each structure was clearly larger when drug was used at 29°C ambient temperatures [5]. A similar change was found in the spinal cord. However, the numbers of damaged cells in spinal cord was relatively smaller than in all previously tested brain structures (**Fig. 7A**).

The number of structurally abnormal cells evaluated in all animals with respect to other parameters revealed maximal and very linear correlation with the number of albumin-positive cells (**Fig. 7B**), suggesting that structural damage directly depends upon the extent of BSCB leakage. A slightly weaker but still strong correlation was found for brain water (**Fig. 7D**), suggesting tight relations between brain edema and structural cell damage. The number of damaged cells was also related to the number of GFAP-positive cells (**Fig. 7C**) and NAcc temperature elevation (**Fig. 7E**).

A representative example of neuronal damage found with Nissl staining is shown in **Fig 8A**. As could be seen, the number of distorted neurons among which some are swollen and some are shrunken after METH exposure were significantly higher in the METH-29°C (c) as compared to METH-23°C groups (b). These images were clearly different from those obtained from in control, saline-treated rats (a). General sponginess, vacuolation and edema could also be seen in the neuropil that is most marked in the 29°C group as compared to the 23°C group following METH administration. Nissl stained neurons also showed elongation and damage to axons as well as dendrites (c). Acute structural abnormalities of spinal cord tissue with myelin degenerations in the axons and dendrites were also found at the ultrastructural level (**Fig. 8B**). Thus, vesiculation of myelin is common and prominent after acute METH exposure and it is much stronger, when the drug is administered at 29°C, inducing more robust changes in all physiological parameters. Many myelinated axons or dendrites showed structural damages including degeneration, swelling or membrane disruption as compared to the control group (Fig. 8B).

DISCUSSION

The brain, its individual structures, various neurochemical systems and circuits and individual neurons are the primary focus of most studies aimed at discovering neural mechanisms underlying the possible health hazards of addictive drugs. While an inseparable part of the CNS, the spinal cord attracts much less attention and is often viewed as a passive relay, transmitting the ascending and descending neural signals that are essential for proper functioning of the brain and central regulation of behavior and physiological functions. However, in addition to “connecting” the brain with the body, the human spinal cord, which comprises only 2% of CNS volume, contains about 1 billion neurons [33], and damage to these cells result in multiple pathologies and great human suffering. In contrast to human brain, the spinal cord in rats is relatively larger and comprises about 35% of a total CNS volume [34, 35]. Similar to the brain, which is reliably protected by the BBB from potentially damaging influences from the body, the BSCB exquisitely regulates transport of water and water-soluble substances into and out of spinal cord tissue, thus providing optimal functioning in this part of the CNS and protecting it from potentially toxic influences [see 10 for review].

To our knowledge, this study is the first to report robust leakage of the BSCB in rats during acute METH intoxication. This effect assessed by intra-tissue entry of EB and albumin immunoreactivity was strongly dependent upon drug-induced brain temperature elevation and was greatly enhanced when METH was used at warm environmental temperatures when brain and body hyperthermia reached clearly pathological values (40-41°C). Although these

data generally match those found in the brain, the number of albumin-positive cells was slightly but significantly lower than that found in the brain (see Fig. 1). A similar tendency was also evident in concentrations of EB. Therefore, it appears that METH induces a generalized barrier leakage, equally present in the brain and spinal cord.

While the cellular and molecular mechanisms underlying increased permeability of the BBB and BSCB remain unclear, our present data suggest that drug-induced hyperthermia could be a critical factor. Although both groups of rats in this study received an equal dose of METH and, therefore, were exposed to the same metabolic impact, both the leakage of the BSCB and temperature elevation were much larger when the drug was administered at warm vs. standard ambient temperatures. Importantly, these parameters tightly correlated within the entire animal population (see Fig. 1D and F). Although our previous study that employed anesthetized animals passively warmed to different brain temperatures, revealed that temperature *per se* is a significant factor affecting BBB permeability [36], hyperthermia is also an integral index of METH-induced metabolic activation. Therefore, in addition to temperature *per se*, other metabolic factors definitely contribute to METH-induced BSCB breakdown. Consistent with this view, BBB leakage during METH intoxication was two-three fold larger than that occurring in anesthetized rats passively warmed to the same brain temperatures [36]. Although NAcc temperature was about 0.8-1.0°C larger than muscle temperature, drug-induced changes in both parameters were very similar, with regression lines parallel to each other (Fig. 1B). Therefore, a tight correlation of neurochemical parameters with brain temperature is equally valid for body temperature.

Similar to that in brain structures, METH intoxication induced acute glial activation in the spinal cord as evidenced by increased numbers of GFAP-positive cells. While changes in this parameter were slightly smaller than in the brain, the pattern of changes was very similar and glial activation was tightly correlated with BSCB leakage and brain hyperthermia (see Fig. 4). Interestingly, despite the higher number of glial cells in the spinal cord compared to the brain, GFAP response in spinal cord was slightly lower than in the brain.

Similar to the brain, METH exposure induced water accumulation in spinal cord tissue. We found that the absolute water content in the spinal cord is clearly lower than in the brain and this difference could be important in determining the higher resistance of this part of CNS (compared to the brain) to ischemic and toxic insults and the development of cell pathology. One of the reasons attributed to this higher resistance of the spinal cord is its flexibility. There is simply more space in the spinal canal than in the brain for swelling. Also, the vascular anatomy of the spinal cord is slightly different than that in the brain in terms of presence of collagen fibers within the spinal large vessels. This feature could be another reason that makes the spinal cord more resistant to ischemic and toxic impacts [16, 29].

However, despite lower concentrations of water in spinal cord tissue, METH induced larger increases in this parameter, with an almost a 3% mean change between control and METH-29°C. Therefore, vasogenic edema equally develops during acute METH intoxication in both compartments of the CNS, and the spinal cord appears to be more vulnerable to edema formation despite slightly lower indices of BSCB leakage. Since barrier

leakage is the primary cause of edema, it is not surprising that water content in the spinal cord was tightly related to albumin immunoreactivity, glial activation, and brain temperature elevation (see Fig. 6).

Our previous work [5] revealed the acute METH intoxication also results in appearance of structurally abnormal cells in the cortex, thalamus, hypothalamus, and hippocampus. Although the numbers of structurally abnormal cells were maximal in the cortex and especially in the most ventrally located piriform cortex, such cells were seen in each brain area tested. Moreover, their numbers were consistently greater in rats that received METH at warm ambient temperature and showed extreme hyperthermia. A similar picture, with 23 vs. 29°C progression, was found in the spinal cord, although the number of such abnormal cells was slightly lower than in the brain structures. Similar to the brain, the number of abnormal cells tightly correlated with albumin and GFAP immunoreactivities, the extent of edema, and hyperthermia (see Fig. 7).

Although the distinction between “normal” and “abnormal” cells in Nissl-stained tissue slices could be to some extent arbitrary and visual cell abnormalities do not necessarily mean cellular death or irreversible damage, this analysis suggests that the cell-damaging effects of METH are typical of both the brain and spinal cord. While we believe that in most cases cellular abnormalities could be related to edema and will disappear if the brain samples are taken days after drug insult, some cells appear to be damaged irreversibly and will eventually die. Multiple cellular abnormalities induced by METH in the spinal cord were also clearly evident at the ultrastructural level. Some neurons showed less dark-stained cytoplasm and karyoplasm indicating a serious, long-lasting, and possibly irreversible damage. Further studies using brain and spinal cord tissues taken at extended time intervals after acute METH exposure could clarify whether these changes are reversible or irreversible. Our preliminary studies, in which brain and spinal cord tissues were taken three days after acute METH impact suggest that at least some of the changes are irreversible in nature [Sharma and Kiyatkin, unpublished observations].

The cellular damage seen within the neuropil of the brain or spinal cord are located in the areas showing intense albumin or Evans blue extravasation. This suggests that breakdown of the BSCB and BBB are the primary triggers to induce abnormal cellular microenvironment hostile to the cells. Obviously, glial cells around the perivascular areas and then in perineuronal areas show acute activation after METH exposure and this perineuronal activation of the GFAP is more prominent when the drug was used at warm ambient temperatures that significantly potentiated its hyperthermic effects [21]. These findings indicate that vascular endothelium is highly vulnerable to minor insults, allowing proteins or large molecules to enter into the brain fluid microenvironment. A massive leak and prolonged exposure of intra-brain environment to serum constituents will lead to further neuronal damage. This is evident with the findings that the intensities of GFAP activation, myelin vacuolation and neuronal damages are the most prominent when METH was administered at the same dose but at slightly higher ambient temperatures. While a high temperature *per se* has a potential to irreversibly damage protein structures of living cells, thus triggering a cascade of events eventually resulting in cell death [37, 38], this thermal

damage could be further enhanced by relatively severe edema and ionic shifts in neural tissue.

METH-induced temperature responses reported in this study are based on direct recordings from the NAcc, a ventrally located forebrain structure. Although it is evident that temperature in the spinal cord should be close to that in the brain and not less than in the body core, according to our knowledge this issue was never assessed experimentally and both basal levels and the pattern of their fluctuations in spinal cord temperatures remain unknown. However, it is known that many spinal cord neurons are temperature-sensitive [39, 40] and METH-induced temperature rise could affect their activity and functions. Since high environmental temperature alone has the potential to induce oxidative stress and subsequently cell damage by lipid peroxidation and over-production of free oxygen radicals, the combination of METH and environmental temperature impact makes a greater damage than that in animals exposed to the same drug at standard laboratory conditions.

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LIST OF ABBREVIATIONS

BBB	blood-brain barrier
BSCB	blood-spinal cord barrier
EB	Evans blue
GFAP	glial fibrillary acidic protein
iv	intravenous
METH	methamphetamine
NAcc	nucleus accumbens
sc	subcutaneous

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Figure 1.

Acute METH intoxication results in hyperthermia and robust leakage of the blood-spinal cord barrier. Left panel shows mean ($\pm$ SEM) temperatures in the brain (NAcc) and temporal muscle (A), concentrations of Evans blue (C) and numbers of albumin-positive cells (E) in rats that received saline (control) and METH at 23°C and 29°C ambient temperatures. White bars show the same parameters determined in the brain. Asterisks show values significantly different from control (***, $p < 0.001$) and circles show the difference between 23 and 29°C (000, $p < 0.001$). Right panel shows the relationships between brain and muscle temperatures (B), brain temperature and Evans blue leakage (D) and brain temperature and albumin immunoreactivity. Each graph shows regression equations, coefficients of correlation (r), and regression lines (bold hatched line).

Figure 2.

Schematic diagram of Evans blue leakage within the spinal cord segments (T9-T12) after METH administration at 23°C and 29°C. The control group (saline injection) did not show Evans blue leakage in any segments of the spinal cord except a slight bluish tint over the superficial dorsal surface of the spinal cord. METH given at 23°C resulted in marked extravasations of Evans blue in both dorsal and ventral horns of the spinal cord segments from T9 to T12 segments. In general, T9 segment showed mild blue staining in the dorsal horn laminae I to IV in the T9, T10 and T12 segments. In the T11 segment lamina V was also stained with Evans blue dye together with laminae I to IV. Leakage of Evans blue was also seen in the spinal cord ventral horn after METH treatment at 23°C that was largely confined within the laminae VIII to X in all the spinal cord segments (T9-T12) examined. On the other hand, when METH was administered at 29°C leakage of Evans blue dye leakage was more intense and spread across the wide areas of the cord. Thus, the blue staining was seen in the dorsal horns of the T9 to T12 segments in the laminae I to V. However, the blue staining was much intense in the laminae IV and V in this group. The ventral horn also showed deep blue staining that was seen in within the laminae VII to IX in all the segments (T9-T12).

Figure 3.

Micrographs from representative examples of albumin (A) and glial fibrillary acidic protein (GFAP, B) immunoreactivity in the spinal cord T9 segment after METH administration at 23°C and 29°C as compared to control. METH administration resulted in albumin extravasation in the spinal cord and activation of GFAP immunoreaction that was stronger at 29°C as compared to identical administration of METH at 23°C. The control group exhibited only a few immunolabelled cells with albumin (A.a) and GFAP (B.a). However, albumin labeled neurons (arrows) could be seen following METH administration at 23°C (A.b) and the magnitude and intensity of this was further enhanced at 29°C (A.c). Likewise, METH at 23°C resulted in activation of astrocytes around the microvessels and some perineuronal areas (B.b, arrowheads) and the magnitude and intensity of this GFAP expression was further exacerbated at 29°C (B.c, arrowheads). Thus, several star shaped astrocytes (arrowheads) are seen within the neuropil showing sponginess and edema (*) in this group. Bar = 35 μ m.

Figure 4.

METH intoxication results in acute glial activation, which correlates with the leakage of BSCB and NAcc temperature elevation. A shows differences in the numbers (mean±SEM) of GFAP-positive cells in rats of each group. White bars show the same parameters determined in the brain. Other graphs show the relationships between the number of GFAP-positive cells and concentration of Evans blue (B), albumin immunoreactivity (C), and NAcc temperatures (D). Each graph shows regression equations, coefficients of correlation (r), and regression lines.

Figure 5. METH intoxication results in alterations of ionic homeostasis and water accumulation in spinal cord tissue. Left panel shows changes in tissue water (A, %) and ions (B, Na⁺, K⁺, and Cl⁻, mM/kg) in the spinal cord in animals of each group. Asterisks show significance vs. control and small circles show significance between two METH groups. Right panel shows changes in the same parameters determined in the brain.

Figure 6.

The extent of METH-induced spinal cord edema depends strongly upon leakage of BSCB (A, albumin immunoreactivity), acute glial activation (B, GFAP immunoreactivity), and brain temperature (C, NAcc). Each graph shows regression equations, coefficients of correlation (r), and regression lines.

Figure 7.

Acute cellular abnormalities in the spinal cord induced by METH intoxication. A shows the numbers (mean±SEM) of damaged cells in the spinal cord and selected brain structures in rats of each group. B-E show the relationships between structural cell damage occurred during METH intoxication and several other parameters (B, albumin immunoreactivity; C, tissue water; E, GFAP immunoreactivity; and F, brain temperature elevation).

Figure 8.

Light micrographs of Nissl-stained slices showing neuronal damage (A) and transmission electron micrographs of myelin vesiculation (B) in the spinal cord T9 segment following METH administration at 23°C and 29° C as compared to the control group. Many healthy neurons are present in normal spinal cord segment in a compact neuropil and signs of neuronal distortion or damage are absent (A.a). However, METH administration resulted in several dark and distorted neurons (arrows) some of them are either swollen or shrunken in the edematous neuropil (A.b). The magnitude and intensity of these neuronal cell damages are further exacerbated when METH was administered at 29°C (A.c). Thus, several neurons are degenerated and showed dark cytoplasm and karyoplasm with eccentric nucleolus (arrow). The neuropil is spongy in nature and showed profound expansion (A.c). Ultrastructural analyses of the spinal cord showed myelin changes after METH administration at 23°C (B.b) and 29°C (B.c) as compared to control (B.a). Myelin vesiculation (arrows) and axonal or dendritic swelling representing edema (*) is apparent in METH treated spinal cord at 23°C (B.b). These changes were much more aggravated when METH was administered at 29°C. Degeneration of axons and dendrites are clearly visible in this group. Bar A = 35 μ m; B = 1 μ m.