Published in final edited form as:

Neuroscience. 2009 July 7; 161(3): 926–939. doi:10.1016/j.neuroscience.2009.04.004.

Permeability of the blood-brain barrier depends on brain temperature

Eugene A. Kiyatkin^{a,*} and Hari S. Sharma^{a,b}

^a Behavioral Neuroscience Branch, National Institute on Drug Abuse–Intramural Research Program, NIH, 5500 Nathan Shock Drive, Baltimore, MD 21224, USA

^b Laboratory of Cerebrovascular Research, Department of Surgical Sciences, University Hospital, Uppsala University, Frödingsgatan 12, #28, SE-751 85 Uppsala, Sweden

Abstract

Increased permeability of the blood-brain barrier (BBB) has been reported in different conditions accompanied by hyperthermia, but the role of brain temperature *per se* in modulating brain barrier functions has not been directly examined. To delineate the contribution of this factor, we examined albumin immunoreactivity in several brain structures (cortex, hippocampus, thalamus and hypothalamus) of pentobarbital-anesthetized rats (50 mg/kg, ip), which were passively warmed to different levels of brain temperature (32–42°C). Similar brain structures were also examined for the expression of glial fibrillary acidic protein (GFAP), an index of astrocytic activation, water and ion content, and morphological cell abnormalities. Data were compared with those obtained from drug-free awake rats with normal brain temperatures (36–37°C). The numbers of albumin- and GFAP-positive cells strongly correlates with brain temperature, gradually increasing from ~38.5°C and plateauing at 41–42°C. Brains maintained at hyperthermia also showed larger content of brain water and Na⁺, K⁺ and Cl[−] as well as structural abnormalities of brain cells, all suggesting acute brain edema. The latter alterations were seen at ~39°C, gradually progressed with temperature increase, and peaked at maximum hyperthermia. Temperature-dependent changes in albumin immunoreactivity tightly correlated with GFAP immunoreactivity, brain water, and numbers of abnormal cells; they were found in each tested area, but showed some structural specificity. Notably, a mild BBB leakage, selective glial activation, and specific cellular abnormalities were also found in the hypothalamus and piriform cortex during extreme hypothermia (32–33°C); in contrast to hyperthermia these changes were associated with decreased levels of brain water, Na⁺ and K⁺, suggesting acute brain dehydration. Therefore, brain temperature *per se* is an important factor in regulating BBB permeability, alterations in brain water homeostasis, and subsequent structural abnormalities of brain cells.

Keywords

brain hyperthermia; blood-brain barrier; acute astrocytic activation; edema; structural brain damage

Correspondence: Eugene A. Kiyatkin, MD, PhD; Behavioral Neuroscience Branch, NIDA–IRP, NIH, 5500 Nathan Shock Drive, Baltimore, MD 21224, USA; tel: 410-550-5551; fax: 410-550-5553; e-mail: E-mail: ekiyatki@intra.nida.nih.gov.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Introduction

In vitro studies suggest that high temperature (>40.0°C) has destructive effects on various cells (Iwagami, 1996; Willis et al., 2000), especially prominent in metabolically active brain cells (Chen et al., 2003; Lee et al., 2000; Lin et al., 1991), including neuronal, glial, endothelial and epithelial cells (Bechtold and Brown, 2003; Sharma and Hoopes, 2003). Rapid damage to brain cells has been also documented *in vivo* during extreme environmental warming (Lin, 1997; Sharma et al., 1992) and acute methamphetamine intoxication (Kiyatkin et al., 2007; Sharma and Kiyatkin, 2008), which both result in robust brain hyperthermia, increased permeability of the blood-brain barrier (BBB), acute glial activation, and vasogenic edema (Bowyer and Ali, 2007; Kiyatkin et al., 2007; Shivers and Wijsman, 1998). The integrity of the BBB is also compromised during opiate withdrawal (Sharma and Ali, 2006), intense physical exercise in a warm environment (Watson et al., 2006) and during restraint and forced swim stress (Esposito et al., 2001; Ovadia et al., 2001; Sharma and Dey, 1986)—conditions associated with brain hyperthermia (Kiyatkin, 2005; Nybo et al., 2002). Although all these data implicate brain hyperthermia as a leading factor in BBB leakage and subsequent damage to brain cells, these changes may be also affected by many other factors (i.e., metabolic brain activation, oxidative stress, alterations in cerebral blood flow, hypoxia of different extent) (Cadet et al., 2001; Nybo, 2008; Sharma, 2006), which contribute to alterations in BBB permeability and subsequent structural brain damage (Haorah et al., 2007; Hom et al., 2001; Kaur and Ling, 2008).

The goal of this study was to examine how brain temperature affects BBB permeability by delineating this factor from other possible contributors. All rats equipped with chronic brain, muscle and skin thermocouple probes were placed under general anesthesia (sodium pentobarbital), which in normal conditions induces marked brain and body hypothermia (Kiyatkin and Brown, 2005; Penicaud et al., 1987). Then, their bodies were passively warmed, resulting in graded increases in brain and body temperatures. The brains were taken at the same time after the start of anesthesia and the initiation of body warming, but at different levels of brain temperature (32–42°C), and analyzed for several parameters. Immunohistochemistry was used to quantitatively evaluate intra-brain leakage of albumin, a measure of BBB breakdown, and expression of glial fibrillary acidic protein (GFAP), an index of astrocytic activation (see Sharma and Ali, 2006; Gordh et al., 2006). Brains were also evaluated for water and ion (Na⁺, K⁺, Cl[−]) content and for the presence of morphological abnormalities, using Nissl and Hematoxylin-Eosin staining. To examine structural specificity of brain alterations, these parameters were assessed separately in the cortex, hippocampus, thalamus, and hypothalamus as well as in other specific areas of interest (i.e., choroid plexus). All these parameters were correlated with brain temperatures recorded immediately before animals were sacrificed, and were compared to control data obtained from the same structures in awake, saline-treated rats that had normal brain temperatures.

Experimental Procedures

Animals and Surgery

Data were obtained from 23 male Long-Evans rats (460±50 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. Care was taken to minimize the number of animals used and any possible their suffering.

All animals were implanted with three thermocouple electrodes as previously described (Brown et al., 2003). Animals were anesthetized with Equithesin (3.3 ml/kg i.p. total volume

containing sodium pentobarbital 32.5 mg/kg and chloral hydrate 145 mg/kg) and mounted in a stereotaxic apparatus. Holes were drilled through the skull over the nucleus accumbens (NAcc) shell (1.2 mm anterior to bregma, 0.9 mm lateral to bregma) using the coordinates of Paxinos and Watson (1998). The dura matter was retracted and a thermocouple probe was slowly lowered to the desired target depth (7.4 mm). A 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-locomotor muscle, which is supplied through the carotid artery by the same arterial blood supply as the brain. Because temperature fluctuations in skin and muscle depend upon two variables: the state of vessels (vasoconstriction/vasodilatation) and the temperature of arterial blood, these two locations were important for evaluating the source of brain hyperthermia/hypothermia and its underlying mechanisms (Kiyatkin, 2005). NAcc was chosen as a representative, deep brain structure implicated on senso-motor integration and behavioral regulation (Wise and Bozarth, 1987). This structure has been used in our thermorecording studies of drug self-administration and psychomotor stimulants (see Kiyatkin, 2005, Kiyatkin and Sharma, 2007), and the relations between temperatures in the NAcc and other brain and peripheral locations were previously examined in detail (Bae et al., 2007). All three probes were secured with dental cement to three stainless steel screws threaded into the skull. Rats were allowed three days recovery and two days of habituation (6–8 hrs) before the start of testing.

Experimental Protocol

All tests occurred at 22–23°C ambient temperatures inside a Plexiglas chamber (32×32×32 cm) equipped with four pairs of infrared motion detectors (Med-PC IV, Med Associates, Burlington, VT, USA) placed inside of a light- and sound-attenuating chamber. The infrared detectors were placed at regular intervals on each outer wall of the test chambers at 2.0 cm above the cage floor level. The resulting grid consisted of four beams. A count was recorded whenever a beam was tripped. 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 jugular catheter, thereby allowing a remote, unsignalled iv injection. 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 two groups: Pentobarbital and Control. After ~2 hrs habituation to the testing chamber, control rats (n=5) received a single intraperitoneal (ip) saline injection (0.3 ml), while each rat in the Pentobarbital group (n=17) received a single ip injection of sodium pentobarbital (50 mg/kg; in 0.3 ml saline). Fifteen min after pentobarbital injection, when rats were fully inactive, a warming pad was placed under the rat's body and the fourth thermal probe was inserted ~5 cm into the rectum, allowing continuous monitoring of rectal temperature. With some animals, the pad was pre-warmed and functional and with others it was not, thus allowing different intensities of gradual body warming. Because of different levels of body warming, brain temperature either decreased (with no warming) or increased to a different extent. 90 min after pentobarbital injection (and 75 min after the start of warming in the Pentobarbital group), rats were taken for brain perfusion. In control rats, perfusion was preceded by ip pentobarbital injection, made 90 min after saline administration. Each animal was perfused with cold 4.0% 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 using 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. Then, the brains were removed and kept in the same fixative at 4°C.

Morphological Observations and Measurements

All histochemical and morphological evaluations were done in four brain regions (cortex, hippocampus, thalamus and hypothalamus) and in four separate cortical areas (cingulate, parietal, temporal and piriform) at the level of the diencephalon (3.25 to 3.90 mm posterior from bregma according to Paxinos and Watson, 1998). These regions and cortical areas are shown in Fig. 1. One half of the brain was used for immunochemistry and counts of abnormal cells, while the other half was used for evaluation of water and ion content.

The integrity of the BBB was evaluated based on the number of albumin-positive cells counted separately in anatomically well-defined regions (see Fig. 1), which were identical in all sections from all animals. Immunostaining for albumin was performed on 3 μ m paraffin brain sections using a sheep polyclonal anti-rat albumin antibody (Sigma, USA) and the streptavidin-HRP-biotin technique as reported previously (Kiyatkin et al., 2007; Sharma et al., 1992). The numbers of albumin-positive cells (irrespective of their presence in neurons or glial cells) were counted manually in three consecutive sections in a blind fashion by at least two independent observers; the median value was used for a final calculation.

Acute glial reaction was evaluated based on the number of GFAP-positive cells counted separately in the same brain areas. Immunostaining was performed on 3 μ m-thick paraffin brain sections 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 (see Sharma et al., 1992 for details). The numbers of GFAP-positive cells were counted in the same anatomical regions (see Fig. 1) in a blind fashion.

The same brain areas (see Fig. 1) were also examined for morphologically abnormal cells, using 3- μ m Nissl- and Hematoxylin-Eosin-stained paraffin sections. The criteria of abnormal cells were the presence of the following characteristics: altered (swollen or shrunken) shape, distorted nucleus, chromatolysis, dark neurons and excentric nucleolus. The numbers of cells having one or all the above parameters were counted manually in a blind fashion within the regions identical in each analyzed brain. Although all these indices suggest structural abnormalities, their presence does not mean that the cell is irreversibly damaged. Significant time is necessary for the damaged cells to become dead and verified as dead, using special histochemical techniques (see Bowyer and Ali, 2006).

In addition to qualitative and quantitative analyses in primary brain regions, Nissl and Hematoxylin-Eosin staining was used to evaluate the structural integrity of the choroid plexus, a critical substrate of blood-cerebrospinal fluid barrier. Profound morphological alterations in this structure have been previously shown during both environmental warming (Sharma and Johanson, 2007) and methamphetamine intoxication (Sharma and Kiyatkin, 2009); in both cases epithelial cell damage was temperature-dependent.

The water content was calculated from the differences between dry and wet weights of the sample (Sharma and Cervós-Navarro, 1990) and ion content (Na^+ , K^+ and Cl^-) was measured from the dry weight of the samples as described earlier (Sharma et al., 1998). In brief, brain samples were dissected out, weighed separately and placed in an oven maintained at 90°C for 72 h to obtain dry weight of the samples. The tissues samples were then processed for determination of Na^+ , K^+ and Cl^- using atomic absorption spectrophotometry (Packard Instruments, Dower Grave, IL, USA) with air-acetylene flame. For greater precision, these

measurements were performed for the cortex as a whole (sum of four areas) and thalamus; the analyzed samples were identical in all brains.

Data Analyses

Temperature and movement data were analyzed with 1-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 comparisons of between-site differences in temperature and locomotion. Correlative and regression analyses were used to assess the relationships between temperatures and brain parameters as well as between individual brain parameters. Light microscopy (Zeiss Observer Z.1) combined with Zeiss AxioVision 4.7 image-processing software (Zeiss, Jena, Germany) was used for qualitative and quantitative analyses of immunostaining and structural alterations of brain cells. The areas of interest were analyzed, using images of brain slices obtained with 100 and 200 magnification.

Results

Temperatures

As can be seen in Fig. 2, the procedure of ip pentobarbital injection results in locomotor activation, increase in NAcc temperature, and a rapid drop in skin temperature (A, B, D). However, ~4 min after the injection, locomotion and brain and muscle temperatures begin to progressively decrease, and skin temperature to increase above its baseline. The NAcc-muscle temperature gradient transiently increases after the injection, but is inverted and stably decreases from ~5 min post-injection (C). In contrast, skin-muscle difference transiently decreases immediately after the injection, but robustly increases from ~5 min post-injection. At 15 min, before warming was initiated, all rats were fully inactive (D) and their brain and muscle temperatures were about 0.6–0.7°C lower than the pre-injection baseline (B).

Since the intensity of body warming varied greatly in different animals, temperatures began to gradually diverge after 15 min, resulting in quite different values at the final recording point (90 min), when each rat was taken for perfusion (see Fig. 2E). At this point, NAcc temperatures varied in individual animals from 32.2 to 42.5°C, covering the range of hypothermia (<34°C; n=4), normothermia (34–38°C; n=5) and hyperthermia (>38°C; n=7). Quantitative data on temperatures in each recording location in these three subgroups of animals are shown in Table. NAcc temperatures in control animals injected with saline (n=5) at the final recording point varied within normal values (36.10–37.40; mean 36.67±0.14°C).

BBB permeability

As can be seen in Fig. 3A (left panel), the number of albumin-positive cells is strongly dependent on brain temperature. In the brain as a whole, this number is minimal at normothermic values (34.2–38.0°C), slightly larger in hypothermic values (34.2–32.2°C), and dramatically larger at hyperthermic values (38.0–42.5°C). The increase is evident from ~39°C, progresses at higher temperature, and plateaus at high levels at 41–42°C. As shown in Fig. 3B and Table 1, this dependence is observed of four structures tested, with some between-structure differences. Maximal increases in albumin immunoreactivity are seen in the thalamus and hippocampus, while the cortex and hypothalamus show smaller changes. There are also some differences in albumin immunoreactivity in different cortical areas, with much stronger changes in the piriform cortex (Fig. 3C). In contrast to all other cortical areas, where increases were evident at 38–39°C, the numbers of albumin-positive cells in the piriform cortex were generally higher within the entire temperature range, with robust increases during hypothermia (8.50±2.22 cells vs. 1.80±0.74 cells in normothermia; p<0.01) and maximal increases during

hyperthermia (17.71 ± 2.67 vs. 1.80 ± 0.74 cells, $p < 0.001$). Figure 4 shows examples of albumin immunoreactivity in the piriform cortex during severe hypothermia (b, 32.2°C) and hyperthermia (c, 42.5°C) with respect to normothermia (a, 36.9°C). As can be seen, hypothermia was associated with mild upregulation of albumin-positive cells and weak immunostaining of the surrounding neuropil. During hyperthermia, leakage of albumin was much more pronounced, with larger numbers of positive cells and stronger immunoreactivity of the neuropil. The albumin-positive cells were often distorted in shape and located in regions showing sponginess and edema. These changes were in sharp contrast to normothermic conditions; only occasional albumin immunoreactivity and virtually no albumin-labeled cells were seen in the piriform cortex in this condition.

Statistical analysis (see Table) revealed that the numbers of albumin-immunoreactive cells in each brain structure in normothermic conditions is identical to those in control animals, but is significantly higher both in hypothermia and hyperthermia. However, the degree of change during hyperthermia is much stronger (~ 26 -fold for the brain as a whole) than that in hypothermia (~ 3.9 -fold).

Acute glial activation

As can be seen in Fig. 3A (middle panel), the number of GFAP-positive cells evaluated in the brain as a whole (sum of four structures) strongly depends on brain temperature. This number is minimal during hypothermia and normothermia, but clearly higher during hyperthermia (38.5 – 42.5°C). Similar to albumin, the increase is evident at NAcc temperatures above 38.5°C and the number of GFAP-positive cells is plateaued at high temperatures. As shown in Fig. 3B and Table, this pattern was evident in each of four structures. The numbers of GFAP-positive cells within the entire range of temperatures is maximal in the thalamus, which also shows the greatest increase during hyperthermia. The values are lower in the hippocampus and minimal in the cortex and hypothalamus; these two structures show the weakest increase in GFAP immunoreactivity at high brain temperatures. There are also some differences in GFAP immunoreactivity in different cortical areas, with clearly higher values in the piriform cortex (c). This cortical area has the largest number of GFAP-positive cells within the entire range of NAcc temperatures and shows significant increases both in hyperthermia (11.14 ± 1.06 cells vs. 3.00 ± 0.55 at normothermia, $p < 0.001$) and hypothermia (8.50 ± 2.63 cells vs. 3.00 ± 0.55 , $p < 0.05$). In all other cortical areas there are virtually no GFAP-positive cells during hypothermia; their numbers are gradually increased from $\sim 38^\circ\text{C}$, with a relative stabilization at very high temperatures. As shown in Fig. 4d, only sporadic GFAP-positive cells are found in the piriform cortex in normothermic conditions, whereas more GFAP-positive, star-shaped astrocytes located around microvessels and neurons are visible during extreme hypothermia (e). During extreme hyperthermia, the number of stained astrocytes is clearly larger (f); they are more intensively stained and preferentially located around the small and large microvessels as well as in the neuropil often around morphologically abnormal neurons in the regions showing edema and sponginess.

Statistical analysis (see Table) revealed that the number of GFAP-immunoreactive cells during normothermia was identical to that in control animals, except for higher count in the thalamus. While in each of four structures these numbers are significantly larger during hyperthermia ($p < 0.001$), the thalamus also shows higher values during hypothermia. In this condition, the number of GFAP-positive cells is significantly larger in the thalamus and hippocampus, but the difference is much lower than that for hyperthermia.

Structural cell alterations

As can be seen in Fig. 3A (right panel), morphologically abnormal cells are absent at low and normal temperatures, and their number linearly increases during hyperthermia. In contrast to

albumin- and GFAP-positive cells, whose counts plateau at very high brain temperature, the number of abnormal cells gradually increases as brain temperature increases. As shown in Fig. 3B and Table, this pattern is evident in each of four structures, but the increase is clearly larger in the thalamus compared to all other structures. There are some minor differences in numbers of abnormal cells among cortical structures (c); again, maximal values are seen in the piriform cortex, which also shows few abnormal cells during hypothermia.

Statistical analysis (see Table) revealed that the number of morphologically abnormal cells in each brain structure during normothermia is identical to that in control animals. While all parameters are significantly higher during hyperthermia ($p < 0.001$), the cortex as a whole and hypothalamus show minor increases ($p < 0.05$) during hypothermia. Interestingly, the piriform cortex is the only cortical area, which shows a significant increase in the number of abnormal cells both in hypothermia (7.50 ± 2.36 cells vs. 1.00 ± 0.78 at normothermia; $p < 0.05$) and hyperthermia (15.14 ± 1.99 , $p < 0.001$ vs. normothermia). As depicted in Fig. 4, few Nissl-stained neurons in the piriform cortex show structural abnormalities during extreme hypothermia (h). Some neurons are shrunken, the neuropil appears to be more compact than that in normothermic conditions (g), and mild perivascular edema is clearly seen around the large and small microvessels. During extreme hyperthermia (i), many cells show pronounced degeneration of the cell nucleus and pyknosis, the neuropil is edematous, and most abnormal cells are found in the areas that show sponginess. Morphologically abnormal cells are often present in clusters: a sign of neurodegeneration and brain swelling. Temperature-dependent alterations in brain structure are also evident in other brain structures. As shown in Fig. 5, neural cells in the same deep areas of the parietal cortex are clearly enlarged and neuronal axons are much wider in hyperthermic conditions compared to normothermia. While structural alterations are less evident in neural tissue during extreme hypothermia (c), neuronal somata and axons are slightly shrunken and neuronal membranes are more condensed than those in the samples obtained at normal brain temperature.

In addition to more-or-less qualitative changes seen in different brain structures, robust structural alterations were also found during hyperthermia in the choroids plexus (Fig. 6). In contrast to compact and dense epithelial cells with distinct nuclei at normothermic and hypothermic conditions, marked degeneration with no distinction between individual cells, dense nuclear staining and vacuolization were typical to brains obtained at extreme hyperthermia (41.8°C).

Water and ion homeostasis

As shown in Fig. 7A and Table, water content both in the cortex and thalamus was directly dependent on brain temperature, with an especially strong, linear correlation in the cortex ($r = 0.98$, $p < 0.001$). Cortical water content was lowest during hypothermia, significantly higher during normothermia and maximal during hyperthermia. Within the range of recorded temperatures, cortical water differed within $\sim 4\%$. In the thalamus, water content was clearly higher during hyperthermia, but values at low and normal temperatures were virtually identical. Cortical water content during anesthesia in normothermic conditions was virtually identical to that in control awake animals, but it was significantly higher (edema) in hyperthermia and significantly lower (dehydration) in hypothermia (Table). A similar trend was seen in the thalamus, where water content during anesthesia was lower than in control at low and moderate brain temperatures and similar to control at high temperatures.

Temperature dependence of Na^+ and Cl^- levels generally mimicked that of water (Fig. 7B and D), and was satisfactorily described by a linear correlation, especially in the cortex. However, the absolute values of Na^+ during anesthesia at hypo- and normothermia were lower than in control (see Table), but Cl^- content was significantly higher in the hyperthermic brain during anesthesia than in normothermic control. In contrast to other ions, K^+ levels in anesthetized

animals were lower than in the awake control during hypo- and normothermia, but clearly increased at higher temperatures (Fig. 7C and Table). This hyperthermia-related increase was larger in the cortex than in the thalamus.

Correlations between brain parameters

In addition to correlation between brain temperature and each of 7 brain parameters, these latter parameters were tightly interrelated with each other. As shown in Fig. 8A, there is a very strong, linear correlation between the numbers of albumin- and GFAP-positive cells ($r=0.96$, $p<0.001$)—two parameters reflecting, respectively, the state of BBB permeability and acute glial activation. Although the regression line is close to the line of equality (=identical values; hatched line), suggesting proportional changes in both parameters, few GFAP-positive cells are present when there were no albumin-positive cells.

There is a strong, linear correlation between the numbers of cells showing albumin-(GFAP) immunoreactivity and morphological abnormalities (Fig. 6B). Abnormal cells are absent when there are no albumin (or GFAP)-positive cells and both parameters increase proportionally.

More complex relationships are seen between the numbers of albumin-positive cells and tissue water (Fig. 6C). Moreover, these relationships have some structural specificity. While both in the thalamus and cortex, the numbers of albumin-positive cells directly correlate with water content ($r=0.96$ and 0.89 , respectively; $p<0.001$), this correlation is highly linear in the thalamus within the entire range of changes, but has some divergence in the cortex at values that correspond to extreme hypothermia (see circle in Fig. 6C). Despite the presence of few albumin-positive cells (see also Fig. 3B), cortical water is relatively lower, suggesting that the tight correlation between brain albumin and water, which exists within the entire range of normal and high temperatures, could be distorted at very low temperatures. Similar relationships are found between morphologically abnormal cells and tissue water (Fig. 6D), which tightly correlate in both cortex and thalamus. Similar to albumin, a minor distortion in correlation is found in the cortex in rats during extreme hypothermia (see circle in D). Under these conditions, cortical water is lower than it should be if the correlation is perfectly linear.

Discussion

Although numerous studies implicate brain hyperthermia as a leading factor of, or a significant contributor to, alterations in BBB permeability and brain damage induced by various environmental and drug challenges, multiple perturbations occurring under these conditions (metabolic activation, hypoxia, release of various neuroactive and neurotoxic substances, etc.) make it difficult to delineate the role of temperature in observed brain alterations. This study was designed to resolve this issue by creating the conditions where the influence of brain temperature on various brain parameters could be assessed independent of metabolic contributions. Consistent with literature (Penicaud et al., 1987) and our previous data (Kiyatkin and Brown, 2005), pentobarbital anesthesia resulted in strong brain and body hypothermia, which has two important features. First, temperature decreases in the NAcc were significantly larger than those in the temporal muscle, suggesting metabolic brain inhibition and diminished intra-brain heat production as a primary cause of brain hypothermia. The profound drug-induced decrease in brain temperature was preceded by a transient, injection-related increase, tightly mimicking the pattern of glucose production and utilization, which both transiently increased immediately after the injection but strongly decreased afterwards (Penicaud et al., 1987). Second, decreases in brain and muscle temperature were associated with relative skin warming, suggesting the loss of vascular constriction (vasodilatation) and increased heat loss to the external environment as another contributor to overall brain and body hypothermia. This basic state of central metabolic inhibition coupled with a diminished ability to maintain temperature homeostasis (or a “vegetative state”) allowed brain temperature to be manipulated

by well-regulated body warming. To eliminate a possible influence of a factor of time, brains were taken at the same time after drug injection (90 min), approximately corresponding to the lowest points of hypothermia under conditions of no body warming (Kiyatkin and Brown, 2005). Therefore, we assumed that differences in brain parameters found in this study are determined by differences in final brain temperatures measured in the NAcc. Since temperature changes in brain areas generally correlate with those in the muscle and rectum (Bae et al., 2007), our conclusions could be also generalized to body temperature.

Although the use of an anesthetized animal preparation with passive body warming creates the conditions for dissociating metabolic brain activation and brain temperature (which are tightly interrelated under natural conditions) and we are pretty confident that the differences in analyzed parameters are determined by temperature variations, it does not mean that other factors that are modulated by temperature cannot contribute to changes in BBB permeability. Nevertheless, this paradigm with only one experimenter-controlled variable appears to be best for examining the contribution of temperature *per se* out of metabolic influences.

Temperature dependence of BBB permeability - Edema

The integrity of the BBB in this study was assessed using immunostaining for endogenous albumin, a relatively large plasma protein (molecular weight 59 kDa, molecular diameter 70 nm) that is normally confined to the luminal side of the endothelial cells and is not present in the brain. Thus, the appearance of albumin-positive cells and albumin immunoreactivity in the neuropil indicates a breakdown of the BBB and the intensity of these changes reflects the extent of BBB leakage. While the appearance of albumin in brain tissue is a consequence of increased BBB permeability, the number of albumin-positive cells correlates tightly and linearly with brain concentrations of Evans blue (Sharma and Kiyatkin, 2009), an exogenous dye tracer traditionally used for evaluating BBB leakage.

Our study revealed that the number of albumin-positive cells is strongly dependent on brain temperature. Both in awake and anesthetized conditions at normothermia, there were virtually no albumin-positive cells in the brain (0.55 and 0.23 cells/slice, respectively). However, this parameter gradually increased from ~38.5°C, reaching clearly abnormal levels at 41–42°C. The mean value of albumin-positive cells during hyperthermia was, respectively, 26- or 63-fold higher than in awake control or anesthetized conditions with maintained normothermia. Therefore, brain hyperthermia appears to be a strong factor, eliciting breakdown of the BBB. Importantly, albumin-positive cells and albumin leakage in neuropil appeared in the brain within the range of physiological hyperthermia (38.5–39.5°C), suggesting that increased BBB permeability is not solely pathological, but also a normal physiological phenomenon occurring during various conditions associated with hyperthermia. Such hyperthermia, for example, occurs during copulatory behavior and heroin self-administration (Kiyatkin, 2005). Although temperature dependence of BBB permeability was evident in all brain structures, the cortex and hypothalamus showed smaller changes in albumin immunoreactivity than the thalamus and hippocampus, while the piriform cortex showed the strongest response among tested cortical areas.

It is well established that albumin entry from the peripheral circulation to the brain results in increased tissue water content (vasogenic edema) (Rapoport, 1985; Zlokovic, 2008). This study supports this view by showing that albumin immunoreactivity and tissue water are both strongly temperature-dependent (Fig. 3 and 6C) and tightly correlate with each other, despite a slight deviation found in the piriform cortex at very low temperatures (32–34°C). At these temperatures, water content was low despite the presence of some albumin-positive cells. Therefore, it appears that extreme hypothermia also results in BBB leakage, but “hypothermic” brains appear to be dehydrated compared to “normothermic” brains taken from awake and anesthetized animals (see Fig. 7A). This dissociation between albumin leakage and lower water

content seems unusual, but it could be related to strong decreases in brain ion content found during hypothermia. While albumin leakage during hyperthermia directly correlates with brain ions (see Fig. 7), promoting water entry to the brain, it is much milder during hypothermia and associated with strong decreases in Na⁺ and Cl⁻, thus promoting water flow from the brain to general circulation along osmotic gradients (Verkman, 2002; Zlokovic, 2008). It is unclear, however, whether these disturbances in water and ion homeostasis are related to hypothermia *per se* or pentobarbital's inhibiting action on metabolism (Penicaud et al., 1987). Importantly, brain temperatures never drop below 34–35°C in any physiological conditions and such extreme hypothermia could only be seen during general anesthesia, overdose of powerful sedative drugs or near-lethal environmental cooling.

The evidence of brain dehydration during extreme hypothermia appears to contradict to other findings, suggesting cold injury-induced edema. However, these latter data were obtained either *in vitro* (Mueller et al., 2000; Plesnila et al., 2000), when the influence of other factors is fully eliminated or *in vivo* but hours after a transient low temperature impact (Arican et al., 2006; Chan et al., 1991; Murakami et al., 1997).

Although our data suggest that brain temperature affects BBB permeability in different brain structures, pointing at brain capillary as a possible underlying substrate, profound morphological alterations in the choroid plexus seen in hyperthermic brains (see Fig. 6) indicate that high temperature also affects the blood-cerebrospinal fluid barrier. Since the choroid plexus plays an important role in the active transport of various substances into and out of cerebrospinal fluid (Sharma and Johanson, 2007), robust morphological abnormalities found in this structure during hyperthermia have obvious functional consequences, contributing to the development of brain edema and damage of brain cells. Possible alterations in permeability at the blood-cerebrospinal fluid barrier could explain, at least in part, regional within-structural differences in albumin and GFAP immunoreactivity, water content, and structural changes, especially in regions proximate to the ventricles.

Acute glial reaction, its temperature dependence and relation to BBB leakage

GFAP is an intermediate filament protein that is expressed in glial cells such as astrocytes. Although an increased GFAP immunoreactivity (or astrocytic activation) is usually viewed as an index of gliosis or a relatively slowly developing correlate of neural damage (Finch, 2003; Hausmann, 2003), rapid GFAP expression has been reported under several conditions associated with robust hyperthermia and brain edema (Cervós-Navarro et al., 1998; Gordh et al., 2006; Kiyatkin et al., 2007; Sharma and Ali, 2006). Unusually rapid dynamics and tight association with edema might suggest that acute GFAP expression differs in its underlying mechanisms from much slower astrocytic proliferation that results in formation of glial scars (gliosis; see Seiffert et al., 2006). This rapid GFAP expression could reflect the interaction of antibodies with GFAP somehow released or made available during membrane damage. Thus, binding sites to GFAP could be increased due to high hyperthermia and associated edema rather than proliferation of astrocytes or elevated levels of GFAP proteins that obviously require more time. Since damage of astrocytes and swelling of the astrocytic end foot results in increased binding of GFAP antibodies (Bailey et al., 1977; Bondarenko and Chesler, 2001), this change could reflect acute and possibly reversible damage of glial cells. Since glial cells could uptake albumin (Ivens et al., 2007), which affects the functional state of glial cells (Ivens et al., 2007; Nadal et al., 1995), glial activation could be at least in part a consequence of BBB leakage and albumin accumulation in brain tissue. While this causal link could be important for slowly developing glial abnormalities, its role in acute, temperature-dependent glial activation remains unclear.

The present study confirms that GFAP expression could occur rapidly (>90 min) and is prominently increased during hyperthermia. The temperature dependence of GFAP

immunoreactivity was similar to that for albumin, showing an exceptionally high, linear correlation between these parameters. However, a close correlation between BBB leakage and acute glial activation does not mean casual relationships between these parameters; both of them are determined by brain hyperthermia. In contrast to albumin, GFAP expression was low at hypo- and normothermia, increased from ~39°C, and stabilized at high levels at 41–42°C. Although GFAP expression during hyperthermia was significantly higher than during normothermia in both anesthetized and awake rats, the increase was quantitatively lower than that for albumin (~4-fold for both conditions) because of the presence of scattered GFAP-positive cells in the controls.

Hyperthermia-induced brain cell abnormalities and their relation to BBB leakage

This study confirms multiple *in vitro* observations, suggesting that brain cells are exceptionally sensitive to thermal damage (see Introduction) and demonstrates that the number of structurally abnormal cells strongly depends on brain temperature. A few abnormal cells were found at ~38.5°C, and their numbers gradually increased as temperature rose. While other parameters (albumin, GFAP) showed a plateau at high temperatures, morphological abnormalities linearly increased and peaked at the maximal detected temperature (42.4°C). Similar to other parameters, structural abnormalities occurred relatively quickly and were tightly related to BBB leakage and increased tissue water content (see Fig. 5B and D). Therefore, even with passive warming, morphological damage reflects not only the effect of temperature *per se*, but also BBB leakage and associated edema.

Although temperature dependence of structural abnormalities was evident in each studied structure, unexpectedly, the thalamus showed the strongest response compared to all other structures. The thalamus also showed the largest increase in GFAP-positive cells during hyperthermia and a mild but significant increase during hypothermia. While these latter changes could be related to the large number of glial cells in this structure, the reasons for such selectivity to thermal damage remain unknown. However, this selectivity is not related to absolute temperatures, which follow a dorso-ventral gradient (Horvath et al., 1999; Mellergard and Nordstrom, 1990), with the “normal” thalamic temperatures higher than in the cortex but lower than in the hypothalamus (see Kiyatkin, 2005). Another brain structure that showed robust morphological abnormalities during extreme hyperthermia was the choroid plexus – the basic substrate of the blood-cerebrospinal fluid barrier. Since structural integrity of this barrier is essential for proper transport of water, ions, various neuroactive substances and metabolites between blood and cerebrospinal fluid, damage of this structure could be an important contributor for alterations in brain water and ionic environment, development of edema, and damage of brain cells.

Although virtually no abnormal cells were seen in the brain in normothermic or hypothermic conditions (see Fig. 2A and B), a small but significant effect was found in the hypothalamus and piriform cortex during extreme hypothermia. In contrast to pyknosis and robust degeneration of cell nucleus with clearly edematous neuropil during hyperthermia, during hypothermia some cells in the piriform cortex were shrunken and neuropil was more compact than in normothermic conditions. While it is unclear whether the deep, ventral location or specific functions of the piriform cortex could be responsible for the selectivity of its cellular response, this structure also showed the largest BBB leakage during hyperthermia, mild but significant leakage during hypothermia and was especially prone to structural damage during environmental warming (Sharma and Cervos-Navarro, 1990), temporal lobe epilepsy (van Vliet et al., 2007), kainate (Candelario-Jalil et al., 2001) and urethane intoxications (Thompson and Westerlain, 2001). Although some evidence of more compact neuropil was seen in other structures, these changes were within the normal range and not recognized as abnormal. Nevertheless, cell somata and especially axons were clearly enlarged in different structures

during hyperthermia and slightly shrunken during extreme hypothermia. These alterations had a generalized nature and they could be related to global changes in intra-brain water homeostasis, when tissue water content fluctuated with respect to “normal” levels with increases or decreases in brain temperature.

Perspectives and significance

Similar to *in vitro* preparation, which allows one to examine the role of temperature in modulating the activity, structure and functions of single cells, the paradigm used in this study was instructive for delineating the factor of temperature from other associated contributions *in vivo*. Pentobarbital by inhibiting brain metabolism dissociates brain temperature and metabolic activity, which are tightly interrelated under natural conditions. Within the physiological continuum, brain temperatures fluctuate within relatively wide limits ($\sim 3^{\circ}\text{C}$), being decreased during sleep and increased following salient environmental stimulation and during motivated behavior (see Kiyatkin, 2009 for review). Brain temperatures could also decrease or increase well below or above its normal physiological range by pharmacological drugs that affect metabolism and heat dissipation. Although methamphetamine intoxication also resulted in BBB breakdown, acute glial activation, edema formation, and structural abnormalities of brain cells and these changes were also temperature-dependent (Kiyatkin and Sharma, 2007), they were much more pronounced despite lower hyperthermia than with passive body warming in this study. Therefore, hypothermia is only one of multiple, interrelated factors that determine functional and structural brain alterations, along with metabolic alterations, oxidative stress, changes in cerebral blood flow, etc.

This study revealed that, in addition to a direct adverse effect of high temperature, BBB leakage results in water accumulation in the brain and a significant change in ionic homeostasis, both contributing to thermal damage. Therefore, brain hyperthermia, independently of its cause (i.e., environmental warming, intense physical exercise under adverse environmental conditions, methamphetamine and ecstasy intoxication, etc.) could be a significant factor eliciting BBB leakage, brain edema and structural abnormalities of brain cells. By increasing BBB permeability, brain hyperthermia could also allow more easy entry to the brain from circulation of various biologically active and potentially neurotoxic substances (i.e., endogenous glutamate and some therapeutic drugs) as well as small viruses (i.e., HIV) that are typically retained by the BBB at normal conditions. Thus, this factor could contribute to the unusually high incidence of neuro-AIDS in methamphetamine users and the co-morbidity of this disease with malaria that is characterized by hyperthermic episodes. This study confirms that marked changes in brain homeostasis could occur rapidly, and they become evident to some extent at the levels of physiological hyperthermia. These data are relevant for understanding why hyperthermia potentiates brain damage induced by other adverse impacts and why pentobarbital and other drugs that decrease brain metabolism and lower brain temperature could be neuroprotective, attenuating the extent of this damage. Further studies, utilizing more sophisticated morphological and histochemical techniques, are needed to define the nature and specificity of thermal damage of brain cells. Such studies are important in clarifying the demarcation between reversible physiological change (adaptation) and irreversible pathological change (damage), which results in profound disturbances of physiological functions and behavior. This issue is also crucial for developing adequate therapeutic strategies, which will maximally promote and supplement an organism's natural abilities for defense, restoration, and repair.

Acknowledgments

This study was supported by the Intramural Research Program of NIDA-NIH, the Leoderal Foundation, and 2007 NIDA Distinguished International Scientist Collaboration Award (NIH) awarded to Hari S. Sharma. The authors greatly appreciate technical assistance of P. Leon Brown, David Bae and Michael S. Smirnov (NIDA-IRP) as well as

Mari-Anne Carlsson and Inga Hörte (Uppsala University). We wish to thank Drs. Barry Hoffer and Roy A. Wise for support of this study and the valuable comments on the matter of this manuscript.

Abbreviations

ANOVA	analysis of variance
BBB	blood-brain barrier
C⁻	chloride
GFAP	glial fibrillary acidic protein
K⁺	potassium
Na⁺	sodium
NAcc	nucleus accumbens

References

- Arıcan N, Kaya M, Yorulmaz C, Kalaycı R, Ince H, Kucuk M, Fincancı SK, Elmas I. Effect of hypothermia on blood-brain barrier permeability following traumatic brain injury in chronically treated rats. *Int J Neurosci* 2006;116:1249–126. [PubMed: 17000527]
- Bae DD, Brown PL, Kiyatkin EA. Procedure of rectal temperature measurement affects brain, muscle, skin, and body temperature and modulates the effects of intravenous cocaine. *Brain Res* 2007;1154:61–70. [PubMed: 17466279]
- Bechtold DA, Brown IR. Induction of Hsp27 and Hsp32 stress proteins and vimentin in glial cells of the rat hippocampus following hyperthermia. *Neurochem Res* 2003;28:1163–1173. [PubMed: 12834255]
- Bekey L, Lee JC, Lee GC, Peng GR. Experimental cerebral concussion: an electron microscopic study. *J Neurosurg* 1977;47:525–531. [PubMed: 903805]
- Bondarenko A, Chesler M. Rapid astrocyte death induced by transient hypoxia, acidosis, and extracellular ion shifts. *Glia* 2001;34:134–142. [PubMed: 11307162]
- Bowyer JF, Ali S. High doses of methamphetamine that cause disruption of the blood-brain barrier in limbic areas produce extensive neuronal degeneration in mouse hippocampus. *Synapse* 2006;60:521–532. [PubMed: 16952162]
- Brown PL, Wise RA, Kiyatkin EA. Brain hyperthermia is induced by methamphetamine and exacerbated by social interaction. *J Neurosci* 2003;23:3924–3929. [PubMed: 12736362]
- Cadet JL, Thiriet N, Jayanthi S. Involvement of free radicals in MDMA-induced neurotoxicity in mice. *Ann Med Intern* 2001;152(Suppl 3):IS57–IS59.
- Candelario-Jalil E, Al-Dalain SM, Castello R, Martinez G, Fernandez OS. Selective vulnerability to kainite-induced oxidative damage in different rat brain regions. *J Appl Toxicol* 2001;21:403–407. [PubMed: 11746182]
- Chan PH, Yang GY, Chen SF, Carlson E, Epstein CJ. Cold-induced brain edema and infarction are reduced in transgenic mice overexpressing CuAl-superoxide dismutase. *Ann Neurol* 1991;29:482–486. [PubMed: 1859179]
- Chen YZ, Xu RX, Huang QJ, Xu ZJ, Jiang XD, Cai YO. Effect of hyperthermia on tight junctions between endothelial cells of the blood-brain barrier model in vitro. *Di Yi Jun Da Xue Xue Bao* 2003;23:21–24.

- Esposito P, Cheorghe D, Kendere K, Pang X, Connolly R, Jaconson S, Theodorides TC. Acute stress increases permeability of the blood-brain barrier through activation of brain must cells. *Brain Res* 2001;888:117–127. [PubMed: 11146058]
- Finch CE. Neurons, glia, and plasticity in normal brain aging. *Neurobiol Aging* 2003;24 (Suppl 1):S123–127. [PubMed: 12829120]
- Gordh T, Chu H, Sharma HS. Spinal nerve lesion alters blood-spinal cord barrier function and activates astrocytes in the rat. *Pain* 2006;124:211–221. [PubMed: 16806707]
- Haorah J, Ramirez SH, Schall K, Smith D, Pandya R, Persidsky Y. Oxidative stress activates protein kinase and matrix metalloproteinases leading to blood-brain barrier dysfunction. *J Neurochem* 2007;101:566–576. [PubMed: 17250680]
- Hom S, Egleton RD, Huber JD, Davis TP. Effect of reduced flow on blood-brain barrier transport systems. *Brain Res* 2001;890:38–48. [PubMed: 11164767]
- Horvath TH, Warden CH, Hajos M, Lombardi A, Goglia F, Diano S. Brain uncoupling protein 2: Uncoupled neuronal mitochondria predict thermal synapses in homeostatic centers. *J Neurosci* 1999;19:10417–10427. [PubMed: 10575039]
- Hausmann ON. Post-traumatic inflammation following spinal cord injury. *Spinal Cord* 2003;41:369–378. [PubMed: 12815368]
- Iwagami Y. Changes in the ultrastructure of human cell related to certain biological responses under hyperthermic culture conditions. *Human Cell* 1996;9:353–366. [PubMed: 9183669]
- Ivens S, Kaufer D, Flores LP, Bechmann I, Zumsteg D, Tomkins O, Seiffert E, Heinemann U, Friedman A. TGF- β receptor-mediated albumin uptake into astrocytes is involved in neocortical epileptogenesis. *Brain* 2007;130:535–547. [PubMed: 17121744]
- Kaur C, Ling EA. Blood–brain barrier in hypoxic-ischemic conditions. *Curr Neurovasc Res* 2008;5:71–81. [PubMed: 18289024]
- Kiyatkin EA. Brain hyperthermia as physiological and pathological phenomena. *Brain Res Rev* 2005;50:27–56. [PubMed: 15890410]
- Kiyatkin EA, Brown PL. Brain and body temperature homeostasis during sodium pentobarbital anesthesia with and without body warming in rats. *Physiol Behav* 2005;84:563–570. [PubMed: 15811391]
- Kiyatkin EA, Brown PL, Sharma HS. Brain edema and breakdown of blood-brain barrier during methamphetamine intoxication: Critical role of brain temperature. *Eur J Neurosci* 2007;26:1242–1253. [PubMed: 17767502]
- Lee SY, Lee SH, Akuta K, Uda M, Song CW. Acute histological effects of interstitial hyperthermia on normal rat brain. *Int J Hyperthermia* 2000;16:73–83. [PubMed: 10669318]
- Lin M-T. Heatstroke-induced cerebral ischemia and neuronal damage. Involvement of cytokines and monoamines. *Ann NY Acad Sci* 1997;813:572–580. [PubMed: 9100936]
- Lin PS, Quamo S, Ho KC, Gladding J. Hyperthermia enhances the cytotoxic effects of reactive oxygen species to Chinese hamster cells and bovine endothelial cells in vitro. *Radiat Res* 1991;126:43–51. [PubMed: 1850533]
- Mellergard P, Nordstrom CH. Epidural temperatures and possible intracerebral temperature gradients in man. *Br J Neurosurg* 1990;4:31–38. [PubMed: 2334525]
- Mueller E, Wietzorrek J, Ringel F, Guretzki S, Baethman A, Plesnila N. Influence of hypothermia on cell volume and cytotoxic swelling of glial cells in vitro. *Acta Neurochir Suppl* 2000;76:551–555. [PubMed: 11450089]
- Murakami K, Kondo T, Chan PH. Blood-brain barrier disruption, edema formation, and apoptotic neuronal death following cold injury. *Acta Neurochir Suppl* 1997;70:234–236. [PubMed: 9416332]
- Nadal A, Fuentes E, Pastor J, McNaughton PA. Plasma albumin is a potent trigger of calcium signals and DNA synthesis in astrocytes. *Proc Natl Acad Sci USA* 1995;92:1426–1430. [PubMed: 7877995]
- Nybo L. Hyperthermia and fatigue. *J Appl Physiol* 2008;104:871–877. [PubMed: 17962572]
- Nybo L, Sechter NH, Nielson B. Inadequate heat release from the human brain during prolonged exercise with hyperthermia. *J Physiol* 2002;545:697–704. [PubMed: 12456844]
- Ovadia H, Abramsky O, Feldman S, Weidenfeld J. Evaluation of the effects of stress on the blood-brain barrier: critical role of the brain perfusion time. *Brain Res* 2001;905:21–25. [PubMed: 11423075]

- Paxinos, G.; Watson, C. *The Rat Brain in Stereotaxic Coordinates*. Vol. Fourth. San Diego: Academic Press; 1998.
- Penicaud L, Ferre P, Kande J, Leturque A, Issad T, Girard J. Effect of anesthesia on glucose production and utilization in rats. *Am J Physiol* 1987;252:E365–E369. [PubMed: 3548431]
- Plesnila N, Nuller E, Guretzki S, Ringel F, Staub F, Baethmann A. Effect of hypothermia on the volume of rat glial cells. *J Physiol* 2000;553:155–162. [PubMed: 10673552]
- Rapoport, SI. *Blood-brain Barrier in Physiology and Medicine*. New York: Raven Press; 1976.
- Seiffert G, Schilling K, Steinhauser C. Astrocyte dysfunction in neurological disorders: a molecular perspective. *Nat Rev Neurosci* 2006;7:194–206. [PubMed: 16495941]
- Sharma HS. Hyperthermia-induced brain oedema: Current status and future perspectives. *Indian J Med Res* 2006;123:629–652. [PubMed: 16873906]
- Sharma HS, Ali SF. Alterations in blood-brain barrier function by morphine and amphetamine. *Ann NY Acad Sci* 2006;1074:198–224. [PubMed: 17105918]
- Sharma HS, Cervos-Navarro J. Brain edema and cellular changes induced by acute heat stress in young rats. *Acta Neurochir Suppl (Wein)* 1990;51:383–386.
- Sharma HS, Dey PK. Influence of long-term immobilization stress on regional blood-brain permeability, cerebral blood flow and 5-HT levels in conscious normotensive young rats. *J Neurol Sci* 1986;72:61–76. [PubMed: 2936871]
- Sharma HS, Hoopes PJ. Hyperthermia-induced pathophysiology of the central nervous system. *Int J Hyperthermia* 2003;19:325–54. [PubMed: 12745974]
- Sharma HS, Johanson CE. Blood-cerebrospinal fluid barrier in hyperthermia. *Prog Brain Res* 2007;162:459–478. [PubMed: 17645933]
- Sharma HS, Kiyatkin EA. Rapid morphological brain abnormalities during acute methamphetamine intoxication in the rat: An experimental study using light and electron microscopy. *J Chem Neuroanat* 2009;37:18–32.
- Sharma HS, Westman J, Nyberg F. Pathophysiology of brain edema and cell changes following hyperthermic brain injury. *Progr Brain Res* 1998;115:351–412.
- Sharma HS, Zimmer C, Westman J, Cervos-Navarro J. Acute systemic heat stress increases glial fibrillary acidic protein immunoreactivity in brain. An experimental study in the conscious normotensive young rats. *Neuroscience* 1992;48:889–901. [PubMed: 1630627]
- Shivers RR, Wijsman JA. Blood-brain barrier permeability during hyperthermia. *Prog Brain Res* 1998;11:413–424. [PubMed: 9632944]
- Thompson KW, Wasterlain CG. Urethane anesthesia produces selective damage in the piriform cortex of the developing brain. *Dev Brain Res* 2001;130:167–171. [PubMed: 11675119]
- Van Vliet EA, da Costa Araujo S, Redeker A, van Schalk C, Aronica E, Gorter JA. Brain-blood leakage may lead to progression of temporal lobe epilepsy. *Brain* 2007;130:521–534. [PubMed: 17124188]
- Verkman A. Physiological importance of aquaporin water channels. *Ann Med* 2002;34:192–200. [PubMed: 12173689]
- Watson P, Shirreffs SM, Maughan RJ. Blood-brain barrier integrity may be threatened by exercise in a warm environment. *Am J Physiol* 2005;288:R1689–R1694.
- Willis WT, Jackman MR, Bizeau ME, Pagliassotti MJ, Hazel JR. Hyperthermia impairs liver mitochondrial functions. *Am J Physiol* 2000;278:R1240–1246.
- Wise RA, Bozarth MA. Psychomotor stimulant theory of addiction. *Psychol Rev* 1987;96:469–492. [PubMed: 3317472]
- Zlokovic BV. The blood-brain barrier in health and chronic neurodegenerative disorders. *Neuron* 2008;57:178–201. [PubMed: 18215617]

Fig. 1. Brain sections showing regions and cortical areas used for quantitative analyses of albumin and GFAP immunoreactivities and structural cell abnormalities. 1, cingulate cortex; 2, parietal cortex; 3, temporal cortex; 4, piriform cortex; 5, hippocampus; 6, thalamus; and 7, hypothalamus.

Fig. 2.

Changes in temperature during pentobarbital anesthesia and the effects of body warming. A, B, and D show, respectively, changes in absolute temperature (NAcc, temporal muscle and skin), relative temperature, and locomotion before and for 15 min after the injection of sodium pentobarbital (50 mg/kg, ip). C shows brain-muscle and skin-muscle temperature differentials. Filled symbols show values significantly different from the pre-injection baseline. The effect of the drug was evaluated with one-way ANOVA with repeated measures ($F_{16,271}=107.75$, 47.81 and 16.76 for NAcc, muscle and skin, respectively; $F_{14,239}=27.49$ for locomotion; each $p<0.001$). E shows changes in NAcc temperature during pentobarbital anesthesia in all animals, divided into three groups (hyperthermia, normothermia and hypothermia) according to their

final temperatures. Values from -10 to $+15$ min are means ($\pm$ SD) for all 16 rats and subsequent values (mean $\pm$ SD) are shown separately for rats divided into three groups. Circles after 90 min are individual values of NAcc temperature in each of 16 experimental rats.

Fig. 3.

Temperature dependence of albumin and GFAP immunoreactivity and cellular brain abnormalities. Data are shown for the sum of all tested structures (A; open circles show original data and closed circles show mean averages), individual brain structures (B), and specific cortical areas (C).

Fig. 4.

Original examples of brain slices of the piriform cortex showing albumin immunoreactivity (a–c), GFAP immunoreactivity (d–f) and Nissl staining (g–i) in pentobarbital-anesthetized rats maintained at different brain temperatures (a, d, g: normothermia; b, e, h: hypothermia; c, f, i: hyperthermia). While in normothermic conditions, albumin-labeled cells were largely absent, with only occasional immunoreactivity in the neuropil (a), massive leakage of albumin was seen in the brain taken at extreme hyperthermia (c). There were multiple albumin-positive cells (arrows), which were often distorted and located in areas showing sponginess, edema, and strong immunoreactivity of the neuropil. Upregulation of albumin-positive neurons with mild immunostaining in the surrounding neuropil was seen during extreme hypothermia (b, arrows). In contrast to mild GFAP immunoreactivity in normothermic (d) and hypothermic (e) conditions, many, strongly GFAP-positive astrocytes (arrows) were found during hyperthermia; these cells were preferentially located around the small and large microvessels in the regions showing edema and sponginess. The Nissl staining at normothermic conditions showed a clear nucleus and triangular shaped neurons with clear cell cytoplasm (g, arrows). Pronounced degeneration of cell nuclei and pyknosis and edematous neuropil were observed in the brain taken at hyperthermia (i, arrowheads); most degenerated neurons were seen in areas showing marked sponginess. Although most cortical cells in the brain taken at extreme hypothermia appear to be normal (h, arrow), few showed signs of degeneration, cell body

shrinkage and more compact neuropil than in normothermic brains. Bars in each row are 100 μm .

Fig. 5. Nissl-stained sections from similar areas of the parietal cortex (deep) obtained from rats maintained at different levels of brain temperature (S15=36.05°C; S29=32.30°C; S12=41.80°C). Brain cells during hyperthermia have larger somata and wider axons (arrows) compared to those in normothermia. In contrast, during hypothermia cells are slightly smaller in size, staining is more condensed, and axons are smaller in diameter compared to normothermic conditions (arrows). Bar for each graph is 100 μ m.

Fig. 6. Profound changes in structural integrity of the choroids plexus during extreme hyperthermia. All slices were made over lateral ventricles, stained with Hematoxylin-Eosin, and images are shown with equal magnification (bar=200 μm). In contrast to the healthy structure in normothermic conditions, robust desintegration of epithelial cells and profound vacuolization were typical of hyperthermic conditions.

Fig. 7.

Temperature dependence of tissue water, Na⁺, K⁺, and Cl⁻ shown separately for cortex and thalamus. Horizontal hatched lines show “normal” values evaluated in brains of awake, drug-free rats at normothermia (see Table for quantitative values). Some graphs also show regression lines, regression equations, and coefficients of correlation for the cortex. Each correlation coefficient value is highly significant ($p < 0.001$).

Fig. 8.

Correlative relationships between individual brain parameters: the counts of albumin- and GFAP-positive cells evaluated in the brain as a whole (A), the counts of albumin (GFAP)-positive cells and morphologically abnormal cells (B), the counts of albumin-positive cells and tissue water content (C; evaluated separately in the cortex and thalamus), and tissue water content and amounts of morphologically abnormal cells (D, evaluated separately in the cortex and thalamus). Each graph contains coefficients of correlation and regression lines. Hatched lines in (A) and (B) are lines of equality. Each correlation coefficient value is highly significant ($p<0.001$).

Table
Temperatures and brain parameters in awake and pentobarbital-injected rats maintained by passive warming at different brain temperatures

	Control n=5	Normothermia (34.5–37.2°C) n=5	Hypothermia (32.2–33.8°C) n=4	Hyperthermia (38.5–42.5°C) n=7
Temperature				
NAcc	36.94±0.14	36.34±0.48	32.99±0.31	40.33±0.55
Muscle	35.99±0.16	35.76±0.27	32.62±0.34	39.14±0.53
Skin	34.91±0.21	35.15±0.21	33.14±0.66	38.71±0.63
Rectal	-	36.00±0.71	32.61±0.39	40.50±0.61
Albumin				
Cortex	0.60±0.25	0.50±0.18	2.38±0.52**	10.21±1.80***
Hippocampus	0.60±0.26	0.20±0.20o	1.75±0.25**	16.86±2.99***
Thalamus	0.80±0.37	0.00±0.00o	3.25±0.95**	18.86±3.11***
Hypothalamus	0.20±0.20	0.20±0.20	1.25±0.25**	12.29±2.46***
GFAP				
Cortex	3.40±0.40	1.25±0.22	2.13±0.66	9.57±1.38***
Hippocampus	6.20±0.80	2.80±0.37	5.00±1.00	14.86±3.81***
Thalamus	3.60±0.68	9.00±1.48	14.50±1.26*	31.71±3.89***
Hypothalamus	2.00±0.32	2.00±0.63	3.00±0.41	7.86±0.83***
Abnormal cells				
Cortex	0.80±0.38	0.25±0.19	1.94±0.50*	12.39±2.12***
Hippocampus	0.40±0.25	0.00±0.00	0.00±0.00	11.14±2.09***
Thalamus	1.00±0.32	0.00±0.00	0.00±0.00	27.29±6.14***
Hypothalamus	0.60±0.25	0.40±0.40	1.50±0.29*	10.00±1.63***
Water, %				
Cortex	74.50±0.08	74.38±0.14	73.38±0.08**	76.46±0.28***
Thalamus	75.50±0.06	73.60±0.14o	73.26±0.06	75.53±0.31***
Na+				
Cortex	321.00±2.10	282.80±4.33o	240.40±2.64***	315.33±8.06***
Thalamus	305.60±2.50	222.40±3.97o	210.60±1.79	300.83±23.10***
K+				
Cortex	221.20±2.15	184.80±2.42o	185.60±1.69	238.33±17.01***
Thalamus	210.00±0.89	186.80±2.26o	189.20±1.99	204.33±6.01*
Cl-				
Cortex	152.40±2.32	153.20±2.80	125.60±4.17**	184.50±7.85***
Thalamus	130.80±1.02	147.20±3.38o	127.60±2.71*	166.83±3.08**

Asterisks (in hypothermia and hyperthermia) show values significantly different from normothermia

* p<0.05;

** p<0.01;

p<0.001; Student's t-test; circles (in normothermia) show statistical significance vs. awake, normothermic control (o, p<0.05; Student's t-test).