Brainstem Structures Are Primarily Affected in an Experimental Model of Severe Scorpion Envenomation

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Received August 6, 2013; accepted September 30, 2013

Severe scorpion envenoming (SSE) is more frequent in children and is characterized by systemic dysfunctions with a mortality rate of up to 9%. Recent evidence shows that the central nervous system (CNS) plays a key role in triggering the cascade of symptoms present in SSE. The age-dependent role of the CNS in SSE lethality may be summarized in 3 hypotheses: (1) the shown increased blood brain barrier permeability of infants to the toxins would especially and primarily compromise neurovegetative control areas, (2) the neurons within these areas have high affinity to the toxins, and (3) the neurovascular interaction is such that SSE metabolically compromises proper function of toxin-targeted areas. A pharmacological magnetic resonance imaging paradigm was used to evaluate localized hemodynamic changes in relative cerebral blood volume (rCBV) for 30 min after the injection of TsTX, the most lethal toxin from the venom of the Tityus serrulatus scorpion. The brainstem showed significant rCBV reduction 1 min after TsTX administration, whereas rostral brain areas had delayed increase in rCBV (confirmed by laser Doppler measurements of cortical cerebral blood flow). Moreover, metabolic activity by 14C-2-deoxyglucose autoradiography showed the highest relative increase at the brainstem. To test whether TsTX has high affinity to brainstem neurons, the lateral ventricle was injected with Alexa Fluor 568 TsTX. Although some neurons showed intense fluorescence, the labeling pattern suggests that specific neurons were targeted. Altogether, these results suggest that brainstem areas involved in neurovegetative control are most likely within the primary structures triggering the cascade of symptoms present in SSE.

Key Words: scorpion envenoming; encephalic perfusion; glucose utilization; cerebral blood flow.

The scorpion envenomation syndrome is a serious public health problem with a particularly high prevalence in tropical countries. Global incidence metrics vary widely in the literature, with some reports estimating up to 1 200 000 cases per year and an annual mortality up to 10 000 cases worldwide (Chippaux and Goyffon, 2008). The severity of scorpion poisoning varies significantly depending on the age of the person exposed to the venom, but it is particularly dangerous in children with up to 9% mortality below 5 years of age (Bahloul et al., 2010). The most successful treatment of scorpion poisoning involves antivenom, which binds to and neutralizes the venom, removing it from circulation. However, the antivenom is not readily available, demands special storage conditions, has narrow time-window efficiency, and also requires access to an intensive care unit in the event of anaphylaxis. Depending on the health status and age of the patient, amount of toxin, and body region involved, the consequences of scorpion envenomation can vary from relatively mild peripheral symptoms such as local pain and paresthesia (in about 90% of the cases) to serious systemic dysfunctions including cardiac arrhythmias, gastrointestinal alterations, lung edema, pancreatitis, convulsions, neurological lesions (ie, brain infarcts), coma, and death (Fernandez-Bouzas et al., 2000; Osnaya-Romero et al., 2001). New pharmacological approaches for the treatment of severe scorpion envenoming (SSE) are urgently needed. This work is an important step in suggesting that therapy should target the protection of key structures in the central nervous system (CNS) rather than concentrating all efforts on antivenom serum development.

The marked differences in severity of scorpion envenomation between children and adults (Ismail, 1995; Otero et al., 2004; Sofer and Gueron, 1988) have led to attempts to elucidate the underlying pathophysiological causes. Contrary to the long-standing previous belief that scorpion toxins did not cross the...
blood brain barrier (BBB), thus concluding that the pathological processes encountered in scorpion stings were due to peripheral activity of the venom; recent findings have suggested a major role of the CNS on SSE lethality. Available evidence thus far shows that the toxins are indeed more permeable to the BBB of young animals and, presumably, children. Nunan et al. (2004), working with the most lethal purified toxin from the crude venom of the Tityus serrulatus scorpion (Tityus toxin—TsTX), showed that even when compensating for the difference in body size, age was a critical determinant of the observed faster absorption and slower clearance of TsTX in young (21–22 day old) versus adult rats, resulting in a 3-fold higher concentration of TsTX in the brain of young rats. Additional support to the age-dependent BBB permeability to scorpion venom toxins has been reported for the scorpion Androctonus australis hector (AahII), with a significant amount of neurotoxin crossing into brain parenchyma after a SC injection in the newborn but not in adult mice (Clot-Faybesse et al., 2000). Nevertheless, the findings of higher BBB permeability do not preclude that the distribution of venom in peripheral organs of young animals may also contribute to lethality. It has been suggested that the presence of TsTX in the heart may explain the frequent association between cardiovascular and neurological symptoms observed in children after exposure to scorpion venom (Nunan et al., 2003). However, when very low doses of TsTX were administered directly into the brain ventricles of adult rats, bypassing their less-permeable BBB and therefore with direct effect on the CNS, the symptoms were the same as seen in high-dose SC injections. As expected, no symptoms were observed when the same low doses of the compound were administered IV to adult rats, presumably due to the insufficient delivery into the brain (Mesquita et al., 2003). Using intraparenchymal routes of administration, the effect of TsTX in neuronal excitability, using concomitant electroencephalographic recordings, has been extensively studied by other colleagues (Nencioni et al., 2009; Sandoval and Lebrun, 2003). In addition, experiments using TsTX have shown that phenobarbital and carbamazepine (anticonvulsant drugs that reduce CNS excitability) can alleviate and eventually completely block many symptoms of the SSE, for example: lung edema (Mesquita et al., 2002), cardiac arrhythmia (Guidine et al., 2008b), and overall increase of the survival rates (Guidine et al., 2008a).

Despite the evidence of central effects of scorpion toxins, there is still scarce knowledge about the brain areas primarily targeted by the venom that could result in a lethal outcome. In a study to examine the relationship between the affected brain regions and the systemic symptoms after SC injection of TsTX in weanling rats, previous work from our laboratory showed early epileptiform discharges in the brainstem nucleus of the solitary tract highly correlated with cardiac alterations and a lower survival time (Guidine et al., 2009). This brainstem nucleus was chosen for its importance in regulating cardiorespiratory function, as well as its close proximity to the area postrema, in which a leaky BBB allows easier passage of various blood-borne compounds. However, in spite of the shown temporal correlation between brainstem activity and cardiovascular alterations, these results cannot extrapolate on suggesting the sequence of activation or importance of brain structures involved in SSE.

So far, this introduction has mainly focused on the pharmacokinetics perspective of SSE, regarding a differential organ/area distribution and uptake in the younger rats when compared with adults (Nunan et al., 2003, 2004). From a pharmacodynamics point of view, TsTX binds to site 3 of voltage-gated sodium channels (VGSC), with higher affinity to channels in the activated state (Bosmans and Tytgat, 2007; Catterall, 1977). One important hypothesis being tested in this work is whether the brain areas functionally compromised after weanling rats are submitted to SC injection of TsTX also have neurons directly targeted by the toxin. Although, to our knowledge, there are no studies in the literature showing affinity of TsTX to a particular sodium channel isoform, it would be preferable to avoid inserting developmental issues (ie, age-dependent isoform expression of VGSC) into the experimental design. In fact, it is already known from the literature that specific sodium channel isoforms are present in different developmental periods (Goldin, 1999). Accordingly, the sodium channel isoforms Nav 1.1 and Nav 1.2 are expressed after birth and reach maximum levels during childhood (Beckh et al., 1989). Nav 1.6 has its maximal levels during early postnatal periods and is highly expressed during adulthood (Felts et al., 1997). Moreover, Nav 1.3 peaks at birth but is undetectable in adult rats (Beckh et al., 1989). The fact that adult animals injected ICV with TsTX have the same symptomatology, cause of death, and physiopathological alterations from those 21-day-old rats inoculated SC is quite suggestive that pharmacokinetics (ie, organ distribution and uptake) plays a more important role than pharmacodynamics in the age-dependent role of the CNS in SSE lethality. Thus, in order to avoid confusing results due to CNS maturation, it was deemed preferable to use adult animals, injected ICV, to evaluate the affinity of specific neural substrates to TsTX that might correlate to the signs and symptoms of SSE.

In summary, this work aims to investigate the sequential involvement and possible functional compromise of brain areas after weanling rats are submitted to SC injection of TsTX. In order to account for the SSE symptoms that lead to lethality, our current hypothesis is that toxins would have to compromise proper function of brain areas significantly involved in neurovegetative control. Thus, ideally, in order to validate our hypothesis, the use of pharmacological magnetic resonance imaging (phMRI), showing the sequence in which hemodynamic changes occur in brain areas during SSE, would have to confirm insufficient blood flow accompanied by high metabolic activity (accessed by 14C-2-deoxyglucose autoradiography; 2-DG) in areas primarily involved in cardiorespiratory control. A contrast agent was used to sensitize the acquisition due to changes in relative cerebral blood volume (rCBV). In addition, the neurons within these candidate areas would have to show
affinity to the scorpion toxin (Alexa-568-labeled TsTX), tested on adult rats for reasons explained in the above paragraph. Our results show that only brainstem nuclei attended to all criteria mentioned above.

MATERIALS AND METHODS

Animals. Experiments were performed on male Wistar rats (Harlan UK; n = 50) that were 21 days old on the day of the TsTX injection. The rats were group housed (with their mothers in litter) and acclimatized for 1 week prior to the commencement of the experiment. They were kept on a 12/12-h light/dark cycle, room temperature of 22°C, and a free access to standard rat chow and water. Efforts were made to avoid any unnecessary distress to the animals. All procedures were performed in accordance with United Kingdom’s Animals (Scientific Procedures) Act of 1986 and approved by Ethics Committees in United Kingdom (PIL 7021135) and Brazilian Ethics Committee in Animal Experimentation (CETEA Protocol 47/2007). Additionally, the experimental protocol entitled “Alexa Fluor 568-TsTX” was performed on male Wistar rats, weighting between 250 and 300 g. They were kept in individual cages and acclimatized during 1 week before the beginning of the experiments.

Scorpion venom and drugs. TsTX was isolated from the venom of T. serrulatus, in agreement with the methodology described by Gomez and Diniz (1966) and modified by Sampaio et al. (1983). The lyophilized toxin was resuspended in 500 µl of sterile saline. A known concentration of TsTX, as determined by Hartree, was used to determine the absorbance coefficient at 280 nm: [Protein] (Ag/ml)/A280 = 279 (Hartree, 1972). Further determination of TsTX concentration was performed by a direct reading of samples in the spectrophotometer (Hitachi spectrophotometer, model 2001, Japan). After determining the concentration of protein, the initial pool was stored at −20°C in 50 µl aliquots. All experiments used the same initial pool of TsTX. For experiments using Alexa Fluor 568, the used dose of TsTX was 1.74 µg/µl (ICV). For other experimental groups, TsTX-treated animals were injected SC with a total of 6 mg/kg of this toxin (2 times the LD50; Numan et al., 2001).

Experimental protocols. Experiments were always performed between 10 am and 6 pm, during light phase.

Pharmacological magnetic resonance imaging. For rCBV measurements, 21-day-old rats (n = 16) were randomly assigned to 2 groups, to be injected SC with TsTX (n = 9) or saline (n = 7). All animals were anesthetized with isoflurane (4% induction; 1.8% maintenance) in a 20:80 O2:air mixture and submitted to tail vein cannulation in order to inject the superparamagnetic contrast Endorem (Guerbet Laboratories Ltd, France) by means of an infusion pump (Endorem = 0.048 ml/min—total volume 0.240 ml—in 5 min). The contrast agent Endorem aimed to increase the sensitivity of the rCBV signal changes due to TsTX injection (see Schwartz et al., 2003). A second catheter was inserted SC in the back of the animal to allow the infusion of TsTX or saline without interrupting baseline image acquisition. Physiologic parameters of respiration, blood oxygen saturation, and heart rate were monitored during the scanning. Body temperature was maintained at 37 ± 0.5°C by means of a heated blanket and rectal temperature probe (Harvard apparatus).

Magnetic resonance images were acquired with a 7.0 T horizontal small bore scanner (Varian, Palo Alto, CA) and a custom-built transmit-receive birdcage RF coil with an inner diameter of 40 mm, linked to a LINUX-based control console running VnmrJ acquisition software (v2.3, Varian).

In the rCBV protocol, an anatomical T2-weighted image was acquired using a fast spin-echo multislice sequence with the following parameters: repetition time (TR) = 4000 ms, effective echo time (TE) = 40 ms, field of view (FOV) = 32 × 32 mm, matrix = 128 × 128, voxel size = 0.25 × 0.25 × 0.6 mm, 8 averages. For phMRI time series, 70 whole-brain T2*-weighted images were acquired using a gradient-echo, multi-echo, multislice sequence, with TR = 937.5 ms, TE = 5, 10, and 15 ms (average image; voxel-wise mean of the 3 echoes), FOV = 32 × 32 mm, 40 × 0.6 mm thick contiguous axial slices, matrix = 128 × 128 resulting in voxels of 0.25 × 0.25 × 0.6 mm, in 60 s per scan, with a total scanning time of 70 min. Twenty such images were acquired before Endorem contrast agent injection (period I), followed by 20 volumes after Endorem contrast agent (period II) and the final 30 volumes after the TsTX or saline injection (period III). The scanning time after TsTX (period III, 30 min) was determined as long enough to allow significant effect of toxin to be observed, but not too long for animals to die during the imaging session.

Preprocessing and statistical analyses of images were performed using the SPM8 software (http://www.fil.ion.ucl.ac.uk; 2010). Region of interest (ROI) analysis was performed using SPM’s toolbox MarsBaR (Marseille Boite a Regions d’Interet) and Prism 5 for Mac Os X (version 5.0e, GraphPad Software, Inc.).

Images from the rCBV protocol were first realigned using a least squares approach and a 6-parameter (rigid body) spatial transformation, where each image was aligned to the first image of the time series. The first image of the time series was then coregistered to the high resolution structural scan and the registration parameters used to realign the remaining time series (SPM8). Brain masks, based on the anatomical scans, were generated automatically using a pulse-coupled neural network algorithm (in-house Matlab script based on Chou et al., 2011) and were used in the spatial normalization and in the statistical analysis to exclude nonbrain areas from the process. The realigned and coregistered images were then spatially normalized to a probabilistic atlas for Wistar rats (Valdes-Hernandez et al., 2011) using a 12-parameter spatial transformation (SPM8). Finally, normalized images were Gaussian smoothed using a full-width and half-maximum 3D kernel of 0.75 × 0.75 × 1.5 mm (150% of the voxel size). To calculate the rCBV time courses, we used an in-house Matlab script developed based on the procedures described by Schwartz et al. (2003). This procedure uses the pre- and post-Endorem periods to calculate rCBVs, at the same time removing the drift in signal intensity that occurs due to wash out of the iron particles. The procedure results in 50 time points (after calculation, the 20 volumes pre-Endorem are excluded from the final time course). Statistical parametric maps, describing the variation at the voxel level, were created for each individual rCBV time series, using a general linear model based on the effect of TsTX measured in the laser Doppler experiments (ie, 20 zeroes, corresponding to the pretoxin period, followed by a linear progression from 0 to 1 starting at time point 25 and ending at the last time point). A second-order analysis was performed using the residual individual contrast maps. Group maps, comparing TsTX-treated group with the saline-treated group (2-sample t test, corrected for multiple comparisons, with threshold set at p < .05), were then overlaid into the probabilistic atlas in order to determine anatomical location of areas with significant changes in rCBV. Finally, the SPM toolbox MarsBaR was used to extract rCBV time courses from ROIs addressing the brainstem and the hypothalamus.

Laser Doppler flowmetry. Rats (n = 12) were arbitrarily assigned to 2 groups: SC injected with TsTX (n = 6) or saline (n = 6). All animals were anesthetized with isoflurane (4% induction; 1.8% maintenance) in a 20:80 O2:air mixture and then positioned in a stereotaxic frame (Kopf). After positioning, animals received a local injection of approximately 0.5 ml of lidocaine chloride hydrochloride plus epinephrine (2%) under the skin over the skull followed by a small incision through the midline. After removal of SC tissue and peristem, the parietal bones were thinned (left and right) at stereotaxic coordinates +4.0 mm anteroposterior, and ±3.0 mm lateral to Bregma, according to Paxinos and Watson stereotaxic atlas (Paxinos and Watson, 1986). Special attention was taken not to drill through the bone. Two laser Doppler flowmetry (LDF) probes (Moor Instruments, UK), with outer diameter 0.8 mm, were inserted into the holes and the cerebral blood flow value was continuously displayed on a monitor. Physiologic parameters of respiration, blood oxygen saturation, and heart rate were monitored during the entire process. The signals were digitized and recorded using the Biopac data-acquisition system (Acknowledgment© 3.9 software) connected to a computer. Body temperature was monitored and maintained at 37°C, as before.

Cerebral blood flow was recorded continuously for 55 min. After a baseline period of 10 min, TsTX or saline was injected, after which recording continued through the remaining 40 min or until animal’s death. After acquisition, time series were averaged in 30-s epochs. The mean time of death was measured in...
the TsTX-treated group. Mean blood flow was measured at mean time of death in both groups and statistical analysis (unpaired t test with p < .05) performed using Prism 5 for Mac Os X (version 5.0c, Graphpad Software, Inc).

14C-2-deoxyglucose. Local cerebral glucose use (GU) was measured in nonanesthetized freely moving rats using an experimental procedure previously described by Kelly et al. (2002). Because a semi-quantitative autoradiographic technique was used, we first developed a pilot protocol in order to determine plasma 14C-2-deoxyglucose (14C-2-DG) and glucose concentrations in young rats throughout the experimental period of 45 min. Rats (n = 6) were injected with 150 µCi/kg of 14C-2-DG (2-deoxy-[1-14C] glucose; GE Healthcare UK Limited) via IP route in 180 µl saline over a 10-s period. The animals were decapitated immediately (0 min) or 1, 5, 10, 20, and 45 min later and a terminal blood sample collected by retro orbital. Plasma was prepared by centrifugation and the concentrations of plasma glucose and 14C-2-DG determined by automated enzymatic assay (YSI 2300 STAT Plus glucose machine) and liquid scintillation counting (LS6500 Multi-purpose Scintillation Counter; Beckman Coulter), respectively. Brains were removed rapidly and homogenized in a 5% trichloroacetic acid solution in 0.1M sodium phosphate buffer, as previously described by Meibach et al. (1980). An index of brain uptake (dpm injected in whole brain/dpm of injected isotope x 100) was determined in all animals (Supplementary Figure 1).

For the main experiment, rats (n = 16) were injected SC with 0.1 ml saline (n = 8) or TsTX (n = 8). Five minutes later, each rat was injected with 150 µCi/kg of 14C-2-DG IP in 180 µl saline over a 10-s period. Rats were returned to their cages for 42.5 min and observed. At the end of this period, rats were anesthetized in a perspex chamber containing isoflurane (4%) in a 20:80% O2:air mixture for 2.5 min. At exactly 45 min after isotope injection, the rats were decapitated and a terminal blood sample collected by retro orbital. The concentrations of plasma glucose and 14C-2-DG were determined as described above. Brains were removed rapidly and frozen in chilled isopentane at −40°C for 10 min. The brains were then placed in aluminum foil bags and stored at −80°C until cryosectioned. The brain sections (20 µm) were picked up on glass cover slips, dried on a hotplate (Clifton Hotplate, Progen Scientific, UK) at 60°C for at least 5 min and placed sequentially in an x-ray cassette with a set of precalibrated 14C methyl methacrylate standards (Autoscale Radiographic Standards; GE Healthcare). Autoradiograms of these sections were prepared by exposing the sections with Kodak single-coated Medical x-ray RM Film (Kodak Scientific Imaging Film) inside an x-ray cassette for exactly 7 days before developing. The autoradiograms were placed on the light box (Northern Lights Model R95; Dual Lamp Precision Illuminator) and photographed with Nikon digital SLR camera (D80) equipped by a macro lens.

Densitometric analysis of autoradiograms was carried out using Image J software (Abramoff et al., 2004). Images were first converted to gray scale tiff files and then into cCi/g maps by comparing to the standard curve constructed from precalibrated radioactivity standards (ranging from 40.1 to 1112.8 cCi/g). As only a terminal blood sample was collected from each rat, GU was semi-quantitatively estimated as the ratio of the tissue 14C (cCi/g) in ROIs over tissue 14C (cCi/g) in corpus callosum, as described by Kelly et al. (2002). In our experiment, corpus callosum was selected as the reference region because neuronal activity is minimal in this white matter region. According to Quelven et al. (2004), normalization to the corpus callosum reduces variations, which can be due to small variations in the amount of the injected radioisotope 14C-2-DG pharmacokinetic and sections thickness. The mean GU in each ROI was calculated from 12 to 16 readings (6–8 sections per rat, left and right). All anatomic brain structures were defined with reference to a stereotaxic rat brain atlas (Paxinos and Watson, 1986). Glucose utilization was thus estimated in 6 structures: frontal (motor) cortex (FCx), parietal somatosensory cortex (Sx), caudate putamen (CPu), thalamus (Thal), dorsolateral/lateral region from periaqueductal gray (PAG), and brainstem (BStem), including the nucleus of the solitary tract (NTS). These regions were selected because of their proximity to the ventricular areas (PAG), involvement in the cardiovascular control (BStem, PAG), or as control areas (CPu, Sx, FCx, Thal). GU ratios from TsTX group were then compared with those from control group by means of a Student's unpaired t test where p < .05 was considered significant.

Alexa Fluor 568-TsTX. TsTX was labeled with Alexa Fluor 568 (Molecular Probes, Eugene, Oregon). Initially, 500 µl of a 2 mg/ml solution of TsTX was mixed with 50 µl of a 1M solution of sodium bicarbonate. The resultant solution was then added to the Alexa-568 vial supplied and homogenized at room temperature (protected from light) for 1 h. Hydroxylamine solution (17 µl), supplied with the labeling kit, was added to the homogenized Alexa-TsTX and the final solution was further homogenized for 30 min. Finally, the resultant solution was filtered through a dialysis membrane (Spectra/ Por Mod 132,290), bathed in a PBS solution (pH 7.2). The toxic effect of the TsTX-Alexa Fluor 568 (TsTX-AF568) complex was confirmed by intracerebral injections of 1 µl in mice and subsequent observation of motor behavior (rotation, piloerection, salivation, etc.) and death within 10 min. After labeling, TsTX-AF568 was stored at −20°C in 10 µl aliquots until the beginning of the experiments.

Rats (n = 4; 250–300 g) were anesthetized with thiopental sodium (40 mg/kg; IP) and injected with atropine (20 mg/kg; IP) in order to prevent cardiac arrhythmias and bronchial hypersecretion secondary to the anesthetic’s use. Prophylactic treatment with antibiotics (enrofloxacin, 10 mg/kg, SC) was performed in order to prevent postsurgical infections. The surgical procedures for cannulae implantation were described elsewhere (Guidine et al., 2008c).

Briefly, rats were submitted to surgery for guide cannulae implantation in right lateral ventricle and ECG electrodes. The used coordinates were anteroposterior (AP): −0.9, latero-lateral (LL): −1.5, and dorso-ventral (DV): −2.5 (Paxinos and Watson, 1986). A hypodermic needle (22 G), 10 mm in length and fastened to the skull bone with zinc cement, was used as a guide cannulae. Electrodes for ECG recordings were made with Teflon-coated stainless steel wires (model 7914; A&M Systems). Approximately 1 cm of the Teflon coat of the wire was removed and this uncoated portion was sutured bilaterally in the thoracic musculature through a small incision. The ECG leads traveled underneath the skin and were exteriorized in the head. A reference electrode, made with surgical screws (model no. 19010-00; Fine Science Tools), was fastened to the nasal bone. The electrodes were soldered to a 6-channel phone connector (RJ11-6) and anchored to the cranium with dental acrylic. After the surgical procedures, the animals were conditioned in individual cages, going through a recovery period of at least 5 days.

After the recovery period, animals were injected ICV with AF568-TsTX using a 5-µl Hamilton syringe connected to the injector needle (dental needle, 30 G, 12 mm in length) through a polyethylene tube (PE-10 Intramid; Clay Adams) filled with distilled water, used to drive toxin into ventricular space. A small 1.0-µl bubble was inserted just before loading the injection needle with 1.0 µl TsTX. ECG recordings started 1 min before Alexa-568-TsTX injection and were done during 50 min or until animals’ death. After the recordings, animals were euthanized and injected with 1.0 µl Evans Blue. The brains were harvested, labeled, and kept in 10% formaldehyde for at least 48h, after which they were sliced to a 45-µm thick slices in a vibratome. The slices were mounted on glass slides. After drying, the slides were colored with neutral red and observed in an optical microscope for confirmation of ventricular injection.

RESULTS

rCBV Magnetic Resonance Imaging

All animals survived the recorded time window of 30 min post-TsTX injection. The temporal profiles of rCBV changes (group mean ± SEM) in 2 ROIs, brainstem (A) and thalamus (B), are shown in Figure 1. A decrease in rCBV can be observed in the brainstem within a minute of TsTX injection. The thalamic rCBV increase is observed later, between 10 and 15 min after TsTX injection. The thalamic rCBV correlates nicely to LDF measurements (Fig. 1C; see next section for details). Analysis of phMRI data showed significant and widespread changes of rCBV correlated to the regressor derived from the
Central effects of TTX in weanling rats

Expected effect of TTX in blood flow. Significant increases in rCBV were observed bilaterally throughout the brain (Fig. 2), particularly in the subcortical areas of the striatum, thalamus/hypothalamus, and midbrain, as well as in the anterior and parietal cortices. Significantly decreased rCBV was also observed, mainly in the brainstem and the cerebellum.

Laser Doppler Flowmetry

The TTX SC injection was lethal and had a mean time to death of 41 ± 7 min. In the TTX group, there was a highly significant increase in LD peak flow after TTX when compared with the saline group (respectively, 62 ± 19% vs 0.9 ± 4.8%; p = .0144). Figure 1D shows the percentage change in LDF measured at peak in TTX-treated animals and at 40 min postinjection in the saline-treated group. Corroborating the rCBV thalamic results, changes in cortical LDF were only significant at 10–15 min after TTX SC injection (Fig. 1C).

In addition, a positive correlation (r = .9188) was observed between time to death and percentage change in LDF (ie, animals that died earlier showed smaller changes), which suggests that cortical LDF increase is an unlikely cause of lethality.

14C-2-deoxyglucose

All animals survived to the experimental procedure. After TTX SC injections, all animals presented the expected behavior, described elsewhere (Guidine et al., 2008a,c, 2009). Briefly, a few minutes after toxin injection, rats presented piloerection, tremor, salivation, immobility, and sometimes vocalization. These effects were accompanied by several changes in the brain metabolic activity. Visual observation of autoradiographs indicated an overall widespread increased metabolism (Fig. 3A). Statistically significant increases of cerebral metabolism, as represented by a ratio of radioactivity in an ROI versus corpus callosum, were found in all measured ROIs including FCx, SCx, Thal, CPu, PAG, and BStem (Fig. 3B). Percentage changes, considering saline activity levels at 100%, are shown in Figure 3C.

All animals injected with TTX exhibited increased plasmatic glucose levels: mean plasma glucose ± SD was 14 ± 1 in the TTX group and 8.7 ± 0.5 in the control group (p < .0001; unpaired t test). However, these increases did not appear to confound the results as the radioisotope concentrations in corpus callosum in control and experimental groups were not

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**FIG. 1.** Relative cerebral blood volume (rCBV) time series and laser Doppler measurements. Time series of mean ± SEM signal intensity values (normalized to baseline) in regions of interest of brainstem (A) and thalamus (B) from TTX-injected (red) and saline-injected (blue) rats. There is an immediate acute CBV decrease in the brainstem (A) after TTX injection. rCBV is increased in the thalamus starting approximately 10–15 min after inoculation with TTX (B). Part (C) shows the laser Doppler Flowmetry measurements, whereas part (D) shows the percentage change in blood flow measured by LDF in animals injected with saline (measured at 40 min postinjection) or TTX (measured at peak). A significant (p < .014) increase in blood flow is observed in rats inoculated with TTX (unpaired t test) compared with shams.
Guidine et al. significantly different: in the controls rats, radioactivity in corpus callosum was $71 \pm 4$ and in the TsTX rats $75 \pm 3$ ngCi/g ($p = .09$, unpaired t test).

Alexa Fluor 568-TsTX

Alexa Fluor 568-TsTX labeling is shown for all periventricular areas in Figure 4. Because labeled AF568-TsTX was injected ICV, there was a strong nonspecific fluorescence at the ventricular borders; however, specific neural labeling was also quite evident (see Fig. 4D—confocal 3D image of an AF568-TsTX-labeled neuron). In Figure 4A, it is readily noticeable that not all hippocampal neurons were targeted by AF568-TsTX; in fact, labeling was found in neurons reasonably distant from the ventricular space. Interestingly, the same specific targeted neuronal labeling was observed in the third and fourth ventricle (Figs. 4B and 4C, respectively). Note that even being the farthest area from AF568-TsTX injection, brainstem neurons were labeled more often than other periventricular areas.

DISCUSSION

In this paper, we show that although the SC administration of TsTX to 21-day-old rats produces widespread alterations of cerebral metabolism and blood flow, the sequence of activation and suggestive metabolic compromise points to the brainstem as a primary targeted area responsible for SSE lethality. Using pharmacological MR imaging of regional cerebral blood volume, enhanced with Endorem contrast agent, we confirmed that the brainstem is recruited within the first minute after...
TsTX administration, showing a significant reduction of rCBV. Thalamic structures show an increase in rCBV that are detected later, at approximately 10–15 min after TsTX administration. Additionally, LDF recordings from the cortex show similar onset latency and increased blood flow. However, animals that died early after TsTX injection showed only modest changes in LDF recordings, implying that cortical hemodynamic changes do not seem to contribute to SSE lethality (Fig. 1C). In fact, different from what was observed for brainstem structures, after TsTX injection, there is a physiological correlation between cortical blood flow and underlying cortical activation (data from 14C-2-DG autoradiography). Nevertheless, it is obvious that the overall effect of TsTX in the cardiorespiratory, vascular, and metabolic processes eventually compromise cortical blood flow (Ismail, 1995). According to these results, the temporal profiles of rCBV changes are also in good agreement with previous electrophysiological results from our laboratory (Guidine et al., 2009). Early brainstem rCBV changes match the timing of electroencephalogram (EEG) spikes recorded in the nucleus of the solitary tract, which preceded cardiac arrhythmias and were directly correlated with a shorter survival time after TsTX in young rats. Meanwhile, the later rCBV increases in cortical areas are equally in agreement with abnormal electrographic activity spread throughout the cortex recorded only later after the TsTX administration. Altogether, these data do not endorse the involvement of cortical structures as playing a primary role in SSE lethality. Additionally, it is important to highlight that the LDF technique is unfortunately limited and cannot be directly applied to brainstem recordings without major surgical intervention, which restricted our LDF data to cortical structures. The LDF recordings, however, were also very important to validate phMRI recordings from cortical structures.

Although the temporal profile of rCBV MRI data shows that the brainstem is among the first areas recruited after TsTX injection, changes in blood volume were very heterogeneous in the spatial distribution. In the caudal brain areas, there were prominent rCBV decreases in the cerebellum, in the ventral brainstem, and also in the posterior sensory, retrosplenial, and entorhinal cortical regions. rCBV was increased in the dorsal portions of midbrain as well as throughout the rostral/frontal brain including areas of motor, cingulate, and sensory cortices, as well as subcortical caudate putamen, hypothalamus.
and thalamus. The worst-case scenario for functional compromise of a specific brain area would be a decrease in rCBV accompanied by an increase in brain metabolism—assessed by 14C-2-DG autoradiography. ROI analysis of 14C-2-DG autoradiography shows a widespread increase, after TsTX, in all brain regions with a most pronounced percentage of increase found in brainstem region (56% increase). Notably, animals injected with TsTX presented significantly higher plasma glucose levels (data not shown). Indeed, hyperglycemia is commonly found in cases of SSE (Cusinato et al., 2010; Ismail, 1995). When differences in plasma glucose between the control and experimental animals are encountered, the results must be interpreted with caution (McCulloch et al., 1982a,b). For instance, a moderate hyperglycemia associated with the administration of the dopaminergic agonist apomorphine reduces the 14C-tissue concentration in the CNS by about 30%. Such a reduction in the absolute levels of radioisotope in the CNS could influence the calculated optical density ratios because these ratios are crucially dependent on absolute isotopes concentrations from which they are derived (Kelly and McCulloch, 1983) and could mask the true utilization of glucose by the cells. In our case, however, this is unlikely, as we found no differences between the groups in absolute radioactivity measured in the white matter reference region of corpus callosum.

Given the timing of 14C-2-DG administration (5 min after TsTX), it is worth bearing in mind that these results provide a snapshot of brain energetic status during an early phase of envenomation, approximately 10–15 min after the toxin. During this phase, albeit in a cohort of anesthetized animals, we see decreased rCBV in the brainstem and the beginning of an rCBV increase in the frontal cortical and subcortical brain areas. Therefore, there was an apparent mismatch between (decreased) cerebral blood flow and (increased) metabolism in the brainstem. This goes contrary to conventional physiological processes, which stipulates that increased cerebral metabolism should be accompanied by increased cerebral blood flow, and therefore rCBV. As an example, changes in functional MRI BOLD signal, increases in particular, are thought to result from

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**FIG. 4.** Alexa Fluor 568-TsTX labeling is shown for all periventricular areas. A, AF568-TsTX-labeled neurons in different regions of hippocampus, even in neurons reasonably distant from the ventricular space; not all hippocampal neurons were targeted by AF568-TsTX. The same specific targeted neuronal labeling was observed in the third and fourth ventricle (B and C); part (D) shows a confocal 3D image of an AF568-TsTX-labeled neuron.
altered neural activity and there is a general consensus that hemodynamic changes measured by MRI and brain activity are tightly correlated in the intact brain (Huppert et al., 2009; Tian et al., 2010). It has been extensively demonstrated that increased neuronal activity is accompanied by increased brain metabolism, oxygen consumption, blood flow, and volume (Lauritzen, 2005; Mueggler et al., 2003). Nevertheless, rCBV data need to be carefully interpreted as the actual neurovascular coupling mechanisms have not yet been fully elucidated (Lauritzen, 2005; Logothetis and Pfeuffer, 2004; Logothetis et al., 2001). Indeed, many studies discuss that this functional hyperemia is region dependent (Devonshire et al., 2012; Shin et al., 2006) and can be altered in pathological conditions, such as focal ischemic depolarizations. In fact, a vasoconstrictive form of neurovascular coupling has been described in the literature, and it seems to occur in response to intense neuronal and astrocytic depolarization (Shin et al., 2006). In addition, any extrapolation between the 2-DG and the MRI data must also take into account the possible confound of anesthesia, which was kept particularly light during phMRI, but not in our 2-DG experimental setup. In spite of all these restrictive remarks, the only area in which decreased rCBV was accompanied by high 14C-2-DG autoradiography labeling was the brainstem.

This mismatch could be explained by either a blood redistribution (“blood stealing”) phenomenon, by direct vascular effects of TsTX (eg, vasoconstriction) or by compromising the neurovascular coupling mechanism through TsTX binding to neurons. The first hypothesis is that the blood is diverted from the less-active to the more-active brain regions resulting in a decreased blood volume to the less activated areas (Bressler et al., 2007). This hypothesis is not supported by our data as the time series has shown an earlier decrease in rCBV in the brainstem, followed by a later increase in the frontal brain areas. If the areas of rCBV increases were responsible for “stealing” the blood from brainstem and cerebellum due to their increased demand, it would be reasonable to expect those areas to show earlier rather than delayed activity. Addressing the second hypothesis, a compound such as TsTX may well have a direct effect on brain vasculature through acting on blood vessels and endothelium causing significant alterations of blood flow in the major cerebral arterioles and potentially overwhelming any subtle fluctuations related to the neurovascular coupling. A vasoconstrictive effect of TsTX has indeed been described by Savino and Catanzaro (1985) who demonstrated a long-lasting constriction of isolated tail arteries after perfusion with TsTX and a concomitant shrinkage of the vascular bed in affected areas. Such a flow-decreasing effect of TsTX could perhaps explain our result of decreased rCBV in the brainstem and surrounding regions. However, it is important to highlight that because the dynamics of rCBV recordings from thalamus and cortex were very different from that observed for brainstem structures, it is quite improbable that TsTX is acting directly upon vasomotor physiology. Rather, considering what is known from previous BBB permeability studies from SC TsTX injections (Nunan et al., 2003, 2004), the region-specific hemodynamic (from rCBV images) and metabolic activity maps (14C-2-DG autoradiography), it is reasonable to assume that TsTX is activating neural substrates that, in turn, modulate neurovascular interaction. Whichever the case, a decreased regional blood supply, coupled with an increased metabolic rate in these same regions, could result in a state of relative hypoxia, which could, in turn, lead to a development of cardiorespiratory changes, such as cardiac arrhythmias and lung edema, by acting on the brainstem cardiorespiratory life support centers. The correlation between neurologic lesions (especially medullar) and neurogenic lung edema has been reported in patients, including those suffering from severe scorpion envenomation. Inobe et al. (2000) described a case of lung edema associated with primary medullar hemorrhage. In addition, Bucaretchi et al. (1995) described 5 cases of lung edema secondary to scorpion envenomation (T. serrulatus and Tityus bahiensis) in children under the age of 15 years. It is our opinion that the management of severe cases of scorpion poisoning should aim at lowering brain metabolism and preventing vasoconstriction induced by the venom in order to avoid the relative state of hypoxia suggested by our data.

Hitherto, we discussed about the pharmacokinetics of TsTX. However, to validate our hypothesis that brainstem is primarily involved in SSE, the toxin used in this study must have affinity to neurons from this area. There are 3 major arguments that guided the choice for using adult animals in the Alexa-568-labeled TsTX experiment: (1) The fact that adult animals injected ICV with TsTX have the same symptomatology, cause of death, and physiopathological alterations when compared with 21-day-old rats inoculated SC (this data suggest that pharmacokinetics, ie, organ distribution and uptake, plays a more important role than pharmacodynamics in the age-dependent role of the CNS in SSE lethality). (2) The voltage-gated Na channels (binding site for the TsTX, Catterall, 1977) have stabilized isoform expression in adults animals (Beckh et al., 1989) and thus would be a preferable age to avoid CNS maturation issues in determining the area/region binding of TsTX. (3) The Alexa-568-TsTX conjugate injected SC has a different brain uptake when compared with TsTX alone (thus inserting a pharmacokinetic contaminant to the evaluation of TsTX binding areas) and would also require a prohibitive amount of TsTX to generate the Alexa-568-TsTX conjugate dosage for SC injection. In a previous article, Mesquita et al. (2003), the LD50 for the ICV route of administration was determined by a sigmoidal fitting of a dose response curve to be at LD50 = 0.131 μg of TsTX. However, the full spectrum of pathophysiological alterations (including piloerection, sialorrhoea, EEG epileptiform discharges, lung edema, respiratory arrest, and death) was only observed at a higher dose of 1.74 μg of TsTX (Mesquita et al., 2003). Thus, experimental design was set at using the higher dose of Alexa Fluor 568-TsTX, in adult animals through an ICV route of injection in order to produce less heterogeneous pharmacodynamic results.

Our experiments using confocal microscopy showed AF568-TsTX-labeled neurons in the brainstem, as well in periventricular areas. These findings are in accordance with other studies in the
literature, which have shown labeled toxin in the cerebral parenchyma after SC or IV injection in rodents (Clot-Faybesse et al., 2000; Nunan et al., 2003) and the effect of TsTX in neuronal excitability (Nencioni et al., 2009). It has already been shown that TsTX binds to VGSC, especially in the activated state, delaying their inactivation and increasing the neuron excitability (Barhanin et al., 1982). Furthermore, studies with synaptosomes have shown that TsTX increased overall internal sodium and calcium ion concentrations and glutamate release in an incremental, dose-dependent manner (Massensini et al., 1998). Sanford and Lebrun (2003) showed that a single intrapopocamal injection of TsTX produces long-lasting effects that mimics epileptogenesis induced by pilocarpine status epilepticus, which reproduces the features of human temporal lobe epilepsy (Sanford and Lebrun, 2003). Additionally, experiments using TsTX have shown that carbamazepine, an anticonvulsant drug that binds to VGSCs in its inactivated state (Kuo et al., 1997), can alleviate and eventually completely block many symptoms of the SSE, for example: lung edema (Mesquita et al., 2002), cardiac arrhythmia (Guidine et al., 2008b), and overall increase of the survival rates (Silva et al., 2013; Guidine et al., 2008a). Interestingly, the labeling pattern of ICV-injected AF568-TsTX in adult animals was not as widespread as would be expected from a voltage-gated Na channel–binding protein. Rather, AF568-TsTX seemed to have targeted very specific neurons within the hippocampus, periventricular areas, and brainstem; heavily labeling some neurons while disregarding adjacent cells. Nevertheless, as expected, brainstem areas associated with cardiovascular and respiratory control showed labeled cells within the same regions with reduced rCBV (phMRI experiment) and increased metabolic activity (14C-2-DG autoradiography).

In summary, the current data corroborate to previous evidence that the brainstem plays a pivotal role in the severity of scorpion intoxication and should be considered a priority target for therapeutic intervention. However, it also appears that the syndrome of scorpion intoxication induces complex and widespread metabolic and hemodynamic changes throughout the brain. Regardless of the origin/mekanism of TsTX pathophysiology (central vs peripheral), such changes in brain function should be taken into account when treating severe cases. Treatments targeting the CNS aiming to decrease brain activity and metabolism might play an important role in the management of severe cases of scorpion envenomation.

SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

FUNDING

The authors are grateful to Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and Pró-Reitoria de Pesquisa da UFMG (PRPq/UFMG) for financial support.

ACKNOWLEDGMENTS

The probabilistic atlas for Wistar rats was created by members of the NMD Lab at Tokoh University. The authors are grateful to Dr Aisling Dixon for her helpful editing of the manuscript.

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