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Fluorophilia: Fluorophore-containing compounds adhere non-specifically to injured neurons

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Abstract

Ionic (free) zinc (Zn^{2+}) is implicated in apoptotic neuronal degeneration and death. In our attempt to examine the effects of Zn^{2+} in neurodegeneration following brain injury, we serendipitously discovered that injured neurons bind fluorescein moieties, either alone or as part of an indicator dye, in histologic sections. This phenomenon, that we have termed “fluorophilia”, is analogous to the ability of degenerating neuronal somata and axons to bind silver ions (argyrophilia — the basis of silver degeneration stains). To provide evidence that fluorophilia occurs in sections of brain tissue, we used a wide variety of indicators such as Fluoro-Jade (FJ), a slightly modified fluorescein sold as a marker for degenerating neurons; Newport Green, a fluorescein-containing Zn^{2+} probe; Rhod-5N, a rhodamine-containing Ca^{2+} probe; and plain fluorescein. All yielded remarkably similar staining of degenerating neurons in the traumatic brain-injured tissue with the absence of staining in our sham-injured brains. Staining of presumptive injured neurons by these agents was not modified when Zn^{2+} in the brain section was removed by prior chelation with EDTA or TPEN, whereas staining by a non-fluorescein containing Zn^{2+} probe, N-(6-methoxy-8-quinolyl)-p-toluenesulfonamide (TSQ), was suppressed by prior chelation. Thus, certain fluorophore-containing compounds nonspecifically stain degenerating neuronal tissue in histologic sections and may not reflect the presence of Zn^{2+} . This may be of concern to researchers using indicator dyes to detect metals in brain tissue sections. Further experiments may be advised to clarify whether Zn^{2+} -binding dyes bind more specifically in intact neurons in culture or organotypic slices.

Keywords

Neuronal degeneration; Traumatic brain injury; Fluorescent indicator; Newport Green; Fluoro-Jade; TSQ

1. Introduction

Ischemia-induced neuronal degeneration has been associated with the appearance of histochemically-reactive or “free” zinc (Zn^{2+}) in degenerating neuronal somata (Choi, 1996; Medvedeva et al., 2009; Tonder et al., 1990). Data also indicate that chelation of Zn^{2+} dramatically reduces neuronal loss after ischemia–reperfusion injury in animals (Calderone

et al., 2004; Koh et al., 1996). Therefore, the role of Zn^{2+} in apoptotic cell death has been intensively studied (Bossy-Wetzel et al., 2004; Capasso et al., 2005; Kim et al., 1999; Levenson, 2005; Li et al., 2010). Indeed, the recent demonstration that zinc-buffering therapy yields improved recovery from clinical stroke in humans (Diener et al., 2008) has encouraged the notion that Zn^{2+} signaling pathways in neuronal death are an attractive target for therapeutic intervention.

The present report summarizes a serendipitous finding about the detection of Zn^{2+} by fluorescent probes in degenerating hippocampal neurons in histologic sections after traumatic brain injury (TBI). These observations were confined to hippocampal neurons both because of the abundance of histochemically-reactive Zn^{2+} in the hippocampus (Frederickson and Danscher, 1990; McLardy, 1964; Slomianka, 1992) and the vulnerability of hippocampal neurons (Friede, 1966) to ischemic and TBI-induced degeneration (Clifton et al., 1989; Jenkins et al., 1989; Lowenstein et al., 1992; Royo et al., 2006). Two peculiarities about the staining triggered the current investigation. First, we observed that the staining of serial sections for degenerating neurons with Fluoro-Jade (FJ), a probe considered specific for neuronal degeneration, and Newport Green (NG), a probe considered specific for Zn^{2+} , produced surprisingly high correspondence between the two staining patterns (Hellmich et al., 2006). We were surprised by the high correlation although it was previously noted that the qualitatively high correspondence between N-(6-methoxy-8-quinolyl)-para-toluenesulfonamide (TSQ) and eosinophilic neurons occurred after TBI (Suh et al., 2000) and after *status epilepticus* (Frederickson et al., 1988; Frederickson et al., 1989). Second, the staining for Zn^{2+} by NG was occurring under conditions that would be expected to eliminate specific staining for Zn^{2+} in the cytosol, namely fixation of the sections in ethanol (Frederickson et al., 1987) and immersion of fixed sections in a Coplin staining jar filled with a solution of the fluorescent Zn^{2+} probe, in which the probe could be saturated by incidental Zn^{2+} in solution. Therefore, we hypothesized that, after TBI or pilocarpine-induced seizures, fluorescein (or rhodamine)-containing dyes might bind nonspecifically to injured neurons in fresh frozen or fixed histopathologic sections as much as silver is known to do, thus rendering fluorescein-containing substances ineffective for identifying Zn^{2+} in injured neurons in fresh frozen or fixed histopathologic sections.

2. Results

2.1. Non-specific binding of fluorescein-based indicators to injured neurons

Cell-impermeant Newport Green (NG), a di-2-picolylamine derivative bound to dichlorofluorescein, is a relatively weak-binding albeit selective Zn^{2+} indicator with a K_D for Zn^{2+} of 1 μM . NG stained neurons in the rat hippocampus, ipsilateral to the injury site, following fluid percussion traumatic brain injury (Fig. 1). Due to the absence of NG positive neurons in the sham rat's brains, photos were omitted. Removal of metals with the chelator, TPEN (100 μM or 1 mM; 5 s to 60 min), revealed that the staining was not appreciably diminished by the Zn^{2+} chelation. FJ, a marker of neuronal injury, stained neurons in similar locations of the injured hippocampus (Fig. 2), but there was an absence of FJ+neurons in the sham-operated rat brains. Correspondingly, when TPEN was applied to the brain sections, the FJ+neurons were still present (Fig. 2). Comparison of the structures of FJ and NG shows that both depend on fluorescein for emissions, and simple fluorescein (without a binding moiety) was tested next. Fluorescein also selectively stained the degenerating neurons in the brain tissue obtained from the TBI rats (Fig. 3).

2.2. TSQ staining of degenerating neurons in trauma and post-seizure models

TSQ, a lipophilic Zn^{2+} probe that lacks the fluorescein moiety, stained the mossy fibers of the hippocampus following both sham and TBI (Fig. 4). In the TBI rats, TSQ positive

neurons were mainly pyramidal neurons found in the CA1, 2 and CA3 subregions of the hippocampus. No TSQ-positive neurons were found in the CA1, 2 and CA3 hippocampal subregions of the sham-operated brains. TSQ staining was blocked by prior immersion of sections in the Zn^{2+} chelator, TPEN, in sections of brain from TBI-injured rats (Fig. 4). Briefly, one-minute fixation of sections in 75% ethanol (EtOH) did not interfere with TSQ staining of sections taken from animals that underwent pilocarpine-induced seizures (Fig. 5). We further showed that the removal of Zn^{2+} from the tissue (using TPEN) proceeded in a time-dependent fashion (Fig. 5).

2.3. Neuronal counting after sham or traumatic brain injury

Animals that undergo TBI and are survived for 24 h have injured neurons in the CA1/2 and CA3 regions of the hippocampus. A comparison of multiple fluorophore-containing indicator dyes in the TBI and sham-injured animals confirmed multiple injured neurons detected in the sections obtained from the injured animals while none of the sham-injured animals displayed positive neurons when stained with any of the dyes (Fig. 6). The 30-minute and two-hour (Table 1) survival time points displayed very few, if any, positive neurons, for any of the indicator dyes tested. Both the 8- and 24-hour survival time points showed an average of 30 stained neurons per section in the CA1 subregion while in the CA3 region, there were approximately 15 stained neurons per section (Table 1). Overall, there were no statistically significant differences between the indicator dyes at any of the survival time points.

3. Discussion

These data clearly demonstrate that (i) fluorescein (or rhodamine)-containing compounds, including simple fluorescein and slightly-modified fluorescein (Fluoro-Jade), adhered to degenerating neurons even after fixation and after the removal of any remaining Zn^{2+} by TPEN, and (ii) staining of Zn^{2+} in brain sections with a fluorescein-free probe (TSQ) was blocked by prior Zn^{2+} chelation by TPEN. Thus, we conclude that degenerating neurons nonspecifically bind fluorophore-containing molecules, a phenomenon that we have termed “fluorophilia”. This nonspecific binding appears to be independent of the presence or absence of other ligands (such as Zn^{2+}) for which those molecules may function as ion probes in other environments such as cell culture or organotypic culture.

Fluoro-Jade has been widely accepted as an indicator of neuronal cell death (Schmued et al., 1997; Ye et al., 2001) in experimental models of ischemia (Duckworth et al., 2005; Duckworth et al., 2006), seizures (Riljak et al., 2005), Parkinson’s disease (Bian et al., 2007), TBI (Hallam et al., 2004; Hellmich et al., 2005; Hellmich et al., 2006; Rojo et al., 2011) and retinal injury (Chidlow et al., 2009), even though both the binding moiety and the cellular ligand of injured neurons to which it binds remain unknown (Schmued et al., 1997; Schmued et al., 2005). Since FJ shares the same emission spectrum as most of the Zn^{2+} indicator dyes, it is difficult to double label the same slides to show co-localization. To further explore the FJ to NG correlation, we decided to stain consecutive sections with the different dyes. We found a strong correlation between FJ and the Zn^{2+} indicator dyes (NG, FZ3, RZ3, and TSQ). In this respect, our findings support the work of Stork and Li (2006) who reported the co-localization of the cell viability stain, propidium iodide, with a Zn^{2+} indicator dye, NG (Stork and Li, 2006). We also found high correlation between FJ and the Ca^{2+} indicator dye, Rhod-5N. This may support the finding that the Ca^{2+} indicator dyes are also sensitive to Zn^{2+} (Canzoniero et al., 1997; Cheng and Reynolds, 1998). The absence of stained cells in the sham-injured brains suggests that the dyes are staining a substance (or substances) produced as a consequence of neuronal injury. This may be due, in part, to the influence of stochastic gene expression in individual neurons which may predispose individual hippocampal neurons to degenerate after injury, while others may be able to

survive (Rojo et al., 2011). Of course, neurons dying after brain trauma are likely filling with Ca^{2+} as well as with Zn^{2+} (Medvedeva et al., 2009; Vander Jagt et al., 2009). Based on our neuronal counting data, the process of neuronal cell death occurs between 2 and 8 h following injury, as this neuronal death is detectable only after the injured animal has survived longer than 2 h. This suggests the possibility of a therapeutic window for treatment within the first 2 h after trauma.

Various kinds of cells in normal or pathologic conditions demonstrate an affinity for certain histologic stains. Therefore, acidophilia, eosinophilia, basophilia and argyrophilia are considered to be specific for various conditions. Argyrophilic cells have been classically demonstrated with four separate silver stains as reviewed by Uchihara (2007) and are seen during degeneration in brain and peripheral nervous system (Cizkova et al., 1996; Ghatak et al., 1986; Iizuka et al., 1989) as well as in various types of cancers (Busch et al., 1979; Elangovan et al., 2008; Pillai et al., 2005). To our knowledge, no definitive explanation of the enhancement of silver binding, or of the other “philias” exhibited by cells has been adduced (Schmued, 2001; Ye et al., 2001).

As is true of the argyrophilic stains such as Bielschowsky and Fink-Heimer (Fink and Heimer, 1967; Switzer, 2000 for review), the “fluorophilia” of the stain, FJ, remains unexplained; no explicit mechanism by which the process of neuronal degeneration renders neuronal cytoplasm “sticky” to the fluorescein congeners has been proposed and accepted (Ye et al., 2001). This is also true for rhodamine-based dyes. Regardless of the absence of a definable mechanism, our data demonstrate that each of the fluorescein (or rhodamine) containing indicators fluoresces in degenerating neurons as does FJ, namely by “binding” to material that in this study was uniquely present in degenerating neurons. In the specific case of NG, the fluorescence of which is enhanced by Zn^{2+} binding, it should be noted that the neuron would have a weak fluorescent signal even if enough of the reporter was localized to the somata in the absence of any Zn^{2+} , because unbound NG weakly fluoresces. That weak fluorescence may be nonspecifically enhanced by Zn^{2+} in the staining solution but would not localize to individual neurons.

This study demonstrated that fluorophore-containing compounds appear to adhere non-specifically to injured neurons. We also showed that this neuronal death is detectable only after the injured animal has survived longer than 2 h. The purpose of this paper is meant to advise researchers to use caution in interpreting the results of experiments that use a fluorescent indicator dye (such as for the detection of Ca^{2+} or Zn^{2+}) in sections of tissue, as these results may show injured neurons instead of a neuron containing zinc or calcium. This knowledge can advance the field by raising awareness that the fluorescent indicator dyes, when used in tissue sections, are actually detecting injured neurons instead of performing their intended purpose.

4. Experimental procedures

4.1. Traumatic brain injury model surgical preparation

The Institutional Animal Care and Use Committee of the University of Texas Medical Branch approved all experimental protocols and all experiments conformed to their guidelines on the ethical use of animals. Male Sprague–Dawley (Charles Rivers, Wilmington, MA) rats were anesthetized (4% isoflurane), intubated, mechanically ventilated with 1.5% isoflurane in O_2 :air (20:80) using a volume ventilator (NEMI Scientific; New England Medical Instruments, Medway, MA) and prepared for moderate or sham parasagittal fluid percussion injury (FPI) (Dixon et al., 1987). Rectal and temporalis muscle temperatures were monitored using telethermometers (Physi-temp Instruments, Clifton, NJ) and temperatures were maintained within a range of 37.5 ± 0.5 °C using an overhead lamp

and a thermostatically controlled water blanket (Gaymar, Orchard Park, NY). Rats were placed in a stereotaxic head holder and a midline incision of the skin was performed and the skull was exposed. With the use of a Michele trephine, a craniotomy was performed 1 mm lateral (right) to the sagittal suture, midway between the lambda and bregma points. The bone chip was removed, leaving the dura intact. A modified 20-gauge needle hub was secured in place over the exposed dura with cyanoacrylic adhesive and cemented into place with hygienic dental acrylic. An arterial line was placed in the animal's tail to monitor mean arterial pressure.

4.2. Parasagittal fluid percussion injury (FPI)

Traumatic brain injury was administered by means of an FPI device consisting of a fluid-filled Plexiglas cylinder 60 cm long and 4.5 cm in diameter, one end of which was connected to a hollow metal cylinder housing a pressure transducer (Statham PA856-100, Data Instruments, Acton, MA) and the other end of which was closed by a Plexiglas piston mounted on O rings. The transducer housing was connected to the rat by a plenum tube at the craniotomy site. Each TBI was induced by dropping a 4.8 kg steel pendulum that struck the piston. The height of the pendulum determined the intensity of the injury. The fluid pressure pulse was recorded on an oscilloscope triggered photoelectrically by the descent of the pendulum. Thirty minutes or 2, 8 or 24 h after TBI or sham injury (n=6 at each time point), rats were re-anesthetized with 4% isoflurane, decapitated and their brains were harvested, immediately frozen in powdered dry ice, and stored at -80°C until sectioned.

4.3. Pilocarpine seizure model

Pilocarpine (Sigma, P-6503) was administered to male Sprague-Dawley rats (300 mg/kg, intraperitoneally). Rats were survived for approximately 3 h and underwent seizures for at least 2 h post injection. Rats that did not experience seizures for at least 2 h were excluded from the study. Animals then were deeply anesthetized and decapitated. Brains were harvested and frozen with CO_2 and immediately prepared for cryostat sectioning. Serial fresh-frozen sections were cut horizontally (30 μM thickness) throughout the entire hippocampus, mounted onto clean glass slides and kept at room temperature for staining.

4.4. Zinc, calcium and injured neuron histology

Zinc, calcium and injured neuron staining was assessed in sections (10 μm coronal TBI model; 30 μm horizontal pilocarpine model) that were stained with both cell-permeable and cell-impermeable indicator dyes. Tissue was prepared as described previously (Hellmich et al., 2005; Hellmich et al., 2006). Briefly, the frozen brains were sectioned on a cryostat, thawed on glass slides and dried by gentle air flow. Some sections were fixed by immersion for 1 min in 75% ethanol. Serial adjacent sections were collected (every 15th section allotted for the same indicator) and stained with either: Fluoro-Jade (FJ; an indicator of lethal cell injury), Newport Green (NG; $K_d = 1 \mu\text{M}$; lowest affinity), FluoZin-3 (FZ3; K_d (threshold concentration for in vitro staining)=15 nM; the highest affinity dye), RhodZin-3 (RZ3; $K_d = 65 \text{ nM}$; intermediate affinity), N-(6-methoxy-8-quinolyl)-p-toluenesulfonamide (TSQ; $K_d = 14\text{--}20 \text{ nM}$), long-wavelength calcium indicator rhod-5N tripotassium salt (Rhod-5N; $K_d \text{ Ca}^{2+} = 320 \mu\text{M}$), or fluorescein (F; without a metal ion binding moiety attached). All indicators were purchased from Invitrogen/Molecular Probes, Eugene, OR except FJ (Histo-Chem, Inc., Jefferson, AR). Consecutive sections were stained since the similar excitation wavelengths of the dyes prevented co-labeling of the same section with multiple indicators.

TSQ staining was performed as previously described (Frederickson et al., 1987; Suh et al., 2000). Sections were stained for Zn^{2+} by immersion in a solution of TSQ (4.5 mmol/L) (Molecular Probes, Eugene, OR) in 140 mmol/L sodium barbital and 140 mmol/L sodium acetate buffer (pH 10.5) for 1 min. After brief rinsing in 0.9% saline, TSQ sections were

examined under a conventional compound fluorescence microscope (Leica: exciter, 355 to 375 nm, dichroic beam splitter, 380 nm, barrier, 420 nm long-pass). For the remaining dyes, sections were immersed in a 5 μ M solution (diluted in Milli-Q water) for 4 min while in an airtight slidebox. All fluorescein and rhodamine stained sections were examined using a Pix-Cell Iie imaging system monitor (Arcturus, Mountain View, CA) under either a FITC or Rhodamine filter.

4.5. Neuronal counting

Numbers of FJ, NG, FZ3, RZ3, TSQ, Rhod-5N and F-positive neurons in the CA1 and 2 and CA3 hippocampal subregions in each of the ten slides per rat brain were counted by an operator unaware of the tissue treatment conditions, using a PixCell Iie imaging system (Arcturus-Molecular Devices, Sunnyvale, CA). For each hippocampal subregion, the numbers of positive neurons were averaged to get a total average number of positive neurons per rat which were then averaged within treatment groups (Hellmich et al., 2005). Since sham-injured rats display very few, if any, positive neurons in comparison to rats that have undergone severe FPI, stereologic counting techniques were not used to compare numbers of stained cells in the injured region of the hippocampus.

4.6. N, N, N', Nr-tetrakis-[2-pyridylmethyl]-ethylenediamine (TPEN) zinc chelation experiments

N, N, N', Nr-tetrakis-[2-pyridylmethyl]-ethylenediamine (TPEN), a Zn^{2+} -selective chelating agent, was used to discriminate Zn^{2+} -induced from non- Zn^{2+} -induced fluorescence in hippocampal post-synaptic neurons. Brain sections (either unfixed or fixed for 1 min with 75% ethanol) were immersed in TPEN (100 μ M or 1 mM) for 5 or 15 s or 2, 5, or 10 min followed by a quick rinse with HEPES buffer and 1 minute incubation with TSQ. Sections were imaged as described above.

4.7. Statistical analysis

For neuronal counts, analysis of variance (ANOVA) was used (Statview 5.0, SAS Institute, Cary, NC). Fisher's PLSD at a significance level of 5% was used for post hoc testing.

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Abbreviations

Ca²⁺	(ionic) calcium
CA1, 2 and 3,	<i>Cornu Ammonis regions 1, 2 and 3</i>
F	fluorescein
FJ	Fluoro-Jade
FPI	fluid percussion injury (an experimental model of TBI)
FZ3	FluoZin-3
NG	Newport Green

OCT	optimum cutting temperature (for embedding)
Rhod-5N	rhod-5N tripotassium salt (Ca^{2+} indicator)
TBI	traumatic brain injury
TPEN	N, N, N', Nr-tetrakis-[2-pyridylmethyl]-ethylenediamine
TSQ	N-(6-methoxy-8-quinolyl)-para-toluenesulfonamide
Zn²⁺	(ionic) zinc

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Fig. 1. Effect of TPEN on Newport Green. Newport Green DCF stained brain sections (right hippocampus shown) from traumatic brain injured rat viewed at 4 $\times$ (A) and 10 $\times$ (B). Serial sections from the same rat were treated with TPEN prior to Newport Green DCF staining, viewed at 4 $\times$ (C) and 10 $\times$ (D).

Fig. 2.
Effect of TPEN on Fluoro-Jade. Fluoro-Jade stained brain sections (right hippocampus shown) from traumatic brain injured rats viewed at 4 $\times$ (A) and 10 $\times$ (B). Serial sections from the same rats were treated with TPEN prior to Fluoro-Jade staining, viewed at 4 $\times$ (C), 10 $\times$ (D), 20 $\times$ (E) and 40 $\times$ (F).

Fig. 3. Fluorescein stained brain sections. Fluorescein stained brain sections (right hippocampus shown) from sham-operated control rats viewed at 4 $\times$ (A) and 10 $\times$ (B) and traumatic brain injured rats viewed at 10 $\times$ (C) and 20 $\times$ (D).

Fig. 4. Zinc staining in hippocampus using TSQ. TSQ stained brain sections (right hippocampus shown) from sham injured (A) and traumatic brain injured rats (B) viewed at 4 $\times$. TSQ stained brain section (right hippocampus shown) from traumatic brain injured rat (C) viewed at 4 $\times$. Serial sections from the same rat were treated with TPEN prior to TSQ staining, viewed at 4 $\times$ (D).

Fig. 5.

The effect of brief ethanol fixation on TPEN chelation of TSQ-positive neurons in pilocarpine model. Brain sections either unfixed or briefly fixed in ethanol (EtOH) for 1 min were immersed in TPEN (100 μ M) for 5 s, 15 s, 2 min, 5 min or 10 min followed by TSQ staining.

Fig. 6.

Comparison of hippocampal sections stained with Zn^{2+} indicator dyes. Adjacent sections of rat hippocampus stained with either Fluoro-Jade (FJ), Newport Green (NG), FluoZin-3 (FZ3), RhodZin-3 (RZ3), N-(6-methoxy-8-quinolyl)-p-toluenesulfonamide (TSQ), Rhodamine-5N (Rhod-5N) following sham or traumatic brain injury (TBI). Animals were survived for 24 h and brain sections were counterstained with Nissl stain (cresyl violet). Slides were examined using a PixCell2E Olympus microscope and photos were taken under 4 $\times$ magnification.

Numbers of positive hippocampal neurons from sham and traumatic brain injured rats. Neurons were counted in the CA1 and 2 and CA3 subregions of the dorsal hippocampus in animals that survived for 30-min, 2 h, 8 h or 24 h post sham or TBI. Numbers represent the mean number of indicator-positive neurons counted from 10 sections per animal (n=6) per survival time point (n=4)±SEM.

Table 1

	FJ			NG			FZ3			RZ3			TSQ			Rhod-SN			Fluorescein-only		
	CA1 and 2	CA3	CA1 and 2	CA3	CA1 and 2	CA3	CA1 and 2	CA3	CA1 and 2	CA3	CA1 and 2	CA3	CA1 and 2	CA3	CA1 and 2	CA1 and 2	CA3	CA1 and 2	CA1 and 2	CA3	CA1 and 2
30 min																					
Sham	0.37 ±0.06	0.03 ±0.02	0.13 ±0.05	0.03 ±0.02	0.10 ±0.03	0.03 ±0.03	0.00 ±0.03	0.00 ±0.02	0.00 ±0.03	0.00 ±0.03	0.00 ±0.04	0.00 ±0.02	0.00 ±0.00	0.00 ±0.04	0.02 ±0.02	0.04 ±0.04	0.12 ±0.04	0.04 ±0.02	0.17 ±0.12	0.07 ±0.03	0.12 ±0.04
TBI	0.23 ±0.06	0.10 ±0.05	0.13 ±0.09	0.10 ±0.03	0.23 ±0.08	0.10 ±0.03	0.07 ±0.03	0.00 ±0.03	0.00 ±0.03	0.00 ±0.03	0.02 ±0.05	0.02 ±0.02	0.03 ±0.02	0.12 ±0.12	0.02 ±0.02	0.17 ±0.12	0.07 ±0.03	0.02 ±0.02	0.17 ±0.12	0.07 ±0.03	0.12 ±0.04
2 h																					
Sham	0.37 ±0.10	0.30 ±0.10	0.07 ±0.02	0.03 ±0.03	0.00 ±0.08	0.03 ±0.03	0.00 ±0.03	0.00 ±0.02	0.00 ±0.03	0.00 ±0.03	0.00 ±0.04	0.00 ±0.02	0.00 ±0.00	0.12 ±0.12	0.08 ±0.05	0.10 ±0.05	0.10 ±0.05	0.10 ±0.05	0.00 ±0.00	0.05 ±0.05	0.10 ±0.05
TBI	0.63 ±0.15	0.30 ±0.22	0.20 ±0.07	0.03 ±0.02	4.03 ±1.95	2.90 ±1.38	2.07 ±0.93	1.40 ±0.62	0.03 ±0.03	0.03 ±0.03	0.05 ±0.05	0.02 ±0.02	0.03 ±0.03	0.12 ±0.12	0.02 ±0.02	0.00 ±0.00	0.05 ±0.05	0.02 ±0.02	0.00 ±0.00	0.05 ±0.05	0.10 ±0.05
8 h																					
Sham	0.23 ±0.06	0.13 ±0.04	0.17 ±0.05	0.03 ±0.02	0.43 ±0.12	0.07 ±0.03	0.10 ±0.04	0.04 ±0.04	0.10 ±0.04	0.03 ±0.03	0.00 ±0.03	0.00 ±0.00	0.00 ±0.00	0.10 ±0.10	0.07 ±0.05	0.00 ±0.00	0.03 ±0.03	0.00 ±0.00	0.44 ±0.44	0.03 ±0.03	0.03 ±0.03
TBI	24.50 ±3.69	17.53 ±1.79	25.17 ±3.58	18.20 ±2.63	22.27 ±4.43	16.30 ±2.18	21.50 ±3.62	14.60 ±1.87	28.12 ±3.97	13.05 ±2.38	27.28 ±3.68	14.38 ±1.90	28.77 ±2.06	12.57 ±2.06	14.38 ±1.90	28.77 ±2.06	12.57 ±2.06	14.38 ±1.90	28.77 ±2.06	12.57 ±2.06	12.57 ±2.06
24 h																					
Sham	0.20 ±0.08	0.07 ±0.03	0.30 ±0.06	0.00 ±0.02	0.03 ±0.07	0.00 ±0.03	0.00 ±0.03	0.00 ±0.00	0.02 ±0.02	0.00 ±0.03	0.12 ±0.05	0.07 ±0.03	0.00 ±0.00	0.12 ±0.12	0.07 ±0.05	0.13 ±0.07	0.27 ±0.22	0.13 ±0.07	0.33 ±0.33	0.27 ±0.22	0.27 ±0.22
TBI	39.37 ±7.74	14.53 ±3.17	32.00 ±6.54	11.37 ±2.38	32.93 ±7.21	12.90 ±2.67	34.37 ±7.05	9.97 ±2.03	20.35 ±4.00	8.07 ±1.74	14.65 ±2.92	6.90 ±1.08	17.23 ±4.33	8.65 ±1.90	6.90 ±1.08	17.23 ±4.33	8.65 ±1.90	17.23 ±4.33	8.65 ±1.90	8.65 ±1.90	8.65 ±1.90