Published in final edited form as:

Exp Neurol. 2012 March ; 234(1): 8–19. doi:10.1016/j.expneurol.2011.12.003.

Substantia nigra vulnerability after a single moderate diffuse brain injury in the rat

Daniel R. van Bregt^{a,b}, Theresa Currier Thomas, Ph.D.^{a,c}, Jason M. Hinzman^{a,c,d,e}, Tuoxin Cao^a, Mei Liu, M.D.^c, Guoying Bing, M.D., Ph.D.^c, Greg A. Gerhardt, Ph.D.^{a,c,d,e}, James R. Pauly, Ph.D.^{a,f}, and Jonathan Lifshitz, Ph.D.^{a,c,g}

^aSpinal Cord & Brain Injury Research Center, University of Kentucky College of Medicine, Lexington, KY, USA

^bDepartment of Biology and Biochemistry, University of Bath, Bath, UK

^cDepartment of Anatomy & Neurobiology, University of Kentucky College of Medicine, Lexington, KY, USA

^dMorris K. Udall Parkinson's Disease Research Center of Excellence, University of Kentucky College of Medicine, Lexington, KY, USA

^eCenter for Microelectrode Technology, University of Kentucky College of Medicine, Lexington, KY, USA

^fDepartment of Pharmaceutical Sciences University of Kentucky College of Pharmacy, Lexington, KY, USA

^gDepartment of Physical Medicine & Rehabilitation, University of Kentucky College of Medicine, Lexington, KY, USA

Abstract

Dementia and parkinsonism are late-onset symptoms associated with repetitive head injury, as documented in multiple contact-sport athletes. Clinical symptomatology is the likely phenotype of chronic degeneration and circuit disruption in the substantia nigra (SN). To investigate the initiating neuropathology, we hypothesize that a single diffuse brain injury is sufficient to initiate SN neuropathology including neuronal loss, vascular disruption and microglial activation, contributing to neurodegeneration and altered dopamine regulation. Adult, male Sprague-Dawley rats were subjected to sham or moderate midline fluid percussion brain injury. Stereological estimates indicated a significant 44% loss of the estimated total neuron number in the SN at 28-days post-injury, without atrophy of neuronal nuclear volumes, including 25% loss of tyrosine hydroxylase positive neurons by 28-days post-injury. Multi-focal vascular compromise occurred 1–2 days post-injury, with ensuing microglial activation (significant 40% increase at 4-days). Neurodegeneration (silver-stain technique) encompassed on average 21% of the SN by 7-days post-injury and increased to 29% by 28-days compared to sham (1%). Whole tissue SN, but not striatum, dopamine metabolism was altered at 28-days post-injury, without appreciable gene or protein changes in dopamine synthesis or regulation elements. Together, single moderate diffuse

© 2011 Elsevier Inc. All rights reserved

Corresponding Author: Jonathan Lifshitz, Ph.D. Spinal Cord & Brain Injury Research Center (SCoBIRC) University of Kentucky Chandler Medical Center Office B463, Biomedical & Biological Sciences Research Building 741 S. Limestone St. Lexington, KY 40536-0509 JLifshitz@uky.edu (859) 323-0696 (859) 257-5737 fax.

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.

brain injury resulted in SN neurovascular pathology potentially associated with neuroinflammation or dopamine dysregulation. Compensatory mechanisms may preserve dopamine signaling acutely, but subsequent SN damage with aging or additional injury may expose clinical symptomatology of motor ataxias and dementia.

Keywords

substantia nigra; PCR; concussion; Parkinsons; brain injury

Introduction

Loss of dopaminergic innervation from the substantia nigra (SN) has been implicated in late onset Parkinson's disease (Bernheimer et al., 1973; Hoehn and Yahr, 1967; Hornykiewicz and Kish, 1987; Lees et al., 2009; Shih et al., 2006). Idiopathic post-mortem pathology shows significant cell dropout and loss of pigmentation among a host of other molecular and cellular markers of cell death (Bernheimer et al., 1973; McGeer et al., 1988). This neuropathology has been modeled using neurotoxic lesions of dopaminergic neurons in the SN in rodents and non-human primates (Bove et al., 2005; Javitch et al., 1985; Smith et al., 1993). Additionally, experimental cerebral ischemia results in SN susceptibility to metabolic dysfunction associated with oxidative damage (Hall et al., 1996). Interestingly, the gender bias for Parkinson's disease weighs heavily towards males, with a world-wide ~2:1 bias (Litvan et al., 2007).

Concussive injury occurs in nearly all contact sports, most prominently in boxing and American football. In these athletes, published reports describe the condition colloquially known as 'punch drunk' dating back to the 1920's (Martland, 1928), predating cellular and molecular techniques to describe the disease process (Roberts et al., 1990). Contemporary nomenclature defined this condition as the degenerative disease *dementia pugilistica*, and more recently as chronic traumatic encephalopathy, where onset often occurs after a career in contact sports with repeated blows to the head (Erlanger et al., 1999; Nowak et al., 2009; Smith et al., 2008; Unterharnscheidt, 1995b, 1995a, 1995e, 1995d, 1995c). Within the past decade, several studies have reported patients exhibiting parkinsonian symptoms who have participated in contact sports during their lives (Forstl et al., 2010; McKee et al., 2009). The brains of these athletes exhibit decreased tyrosine hydroxylase expression (Yan et al., 2007), chronic traumatic encephalopathy and regional pallor in the SN (Casson et al., 2006; Forstl et al., 2010; Omalu et al., 2005; Omalu et al., 2006; Omalu et al., 2010), among other neuropathological findings across the brain. The gender bias for brain injury lies predominantly with males, accounting for 80–90% of all injuries (Langlois et al., 2004).

Despite the basal ganglia susceptibility to oxidative damage, the majority of previous experimental brain injury studies have overlooked the SN in their results and discussions. Some reports have quantitatively measured the SN as part of an extensive survey of brain regions in rat, but findings were neither discussed nor further explored (Hicks et al., 1996; Hovda et al., 1991; Yoshino et al., 1991). Only recently has the vulnerability of the substantia nigra been investigated after experimental TBI (lateral FPI), where a 15% unilateral loss of dopaminergic neurons by 1 week progresses to 30% loss by 26 weeks, concomitant with microglial activation only at the acute time point (Hutson et al., 2011). Midline fluid percussion injury (mFPI) in rodents provides a clinically relevant model of diffuse brain injury that reproduces features of human pathology (e.g. axonal injury) and ensuing motor and cognitive deficits (Lifshitz, 2008). Using a single mFPI of moderate severity in the rat, we investigate the initiating histopathological vulnerability of the SN and

quantify damage to the neurovascular unit with the intent to further understand the etiology of neurological conditions involving SN degeneration.

Materials & Methods

Midline fluid percussion brain injury (mFPI)

Adult male Sprague-Dawley rats (350–375g) were subjected to mFPI consistent with methods previously described (Hosseini and Lifshitz, 2009; Lifshitz et al., 2007). Final animal numbers are indicated in the results section for each study. Briefly, rats were anesthetized with 5% isoflurane in 100% O₂ and maintained at 2% via nose cone. During surgery, body temperature was maintained with a Deltaphase® isothermal heating pad (Braintree Scientific Inc., Braintree, MA). The animal was secured in a stereotaxic frame (Kopf Instrument, Tujunga, CA) and a midline scalp incision exposed the skull. A 4.8-mm circular craniotomy was performed (centered midway between bregma and lambda on the sagittal suture) without disrupting the underlying dura or superior sagittal sinus. An injury cap was fabricated from the female portion of a Luer-Loc needle hub, which was cut, beveled, and scored to fit within the craniotomy. A skull screw was secured in a 1-mm hand-drilled hole into the right frontal bone. The injury hub was affixed over the craniotomy by applying cyanoacrylate gel and methyl-methacrylate (Hygenic Corp., Akron, OH) around the injury hub and screw. The incision was sutured at the anterior and posterior edges and topical Lidocaine ointment was applied. Animals were returned to a warmed holding cage and monitored until ambulatory (approximately 60–90 min).

For injury induction, animals were re-anesthetized with 5% isoflurane 60–90 min after surgery. The dura was inspected through the injury-hub assembly, which was then filled with normal saline and attached to the male end of the fluid percussion device (Custom Design and Fabrication, Virginia Commonwealth University, Richmond, VA). A brain injury of moderate severity (1.9–2.0 atm) or sham injury was administered by releasing the pendulum onto the fluid-filled cylinder as reflexive responses returned. Animals were monitored for the presence of a forearm fencing response and the return of the righting reflex as indicators of injury severity (Hosseini and Lifshitz, 2009). After injury, the injury hub assembly was removed *en bloc*, integrity of the dura was observed, bleeding was controlled with Gelfoam (Pharmacia, Kalamazoo, MI), and the incision was stapled. Moderate mFPI animals had righting reflex recovery times greater than 6 minutes and sham-injured animals recovered within 15 seconds. After recovery of the righting reflex, animals were placed in a warmed holding cage before being returned to the vivarium. Experiments were conducted in accordance with NIH and institutional guidelines concerning the care and use of laboratory animals. Adequate measures were taken to minimize pain or discomfort. For this series of studies, no animals died as a result of the brain injury procedures.

Stereology tissue preparation and Giemsa stain

Rats were euthanatized at 7 days or 28 days after brain or sham injury with an overdose of sodium pentobarbital (150 mg/kg, i.p.) and transcardially perfused with 0.9% NaCl followed by 4% paraformaldehyde/0.1% glutaraldehyde in Millonig's buffer. The brains were removed and blocked in the coronal plane (12 mm extent in the rostral-caudal plane) and paraffin processed. Post-processing, brains were embedded, comprehensively sectioned at 30 µm in the coronal plane (Shandon, AS325, Waltham, MA), and wet mounted on 2% gelatin-subbed slides. Every other section was heated, deparaffinized, rehydrated, stained with 10% Giemsa stain (EM Sciences, #26156-01) at 60°C, differentiated with 1% acetic acid, dehydrated, and coverslipped. The histological sections were the same as those previously used to quantify the ventral basal complex (Lifshitz et al., 2007) and primary somatosensory barrel cortex (Hall and Lifshitz, 2010; Lifshitz and Lisembee, 2011).

Design-based stereology

Design-based stereological estimates of neuronal nuclear volume, neuronal number, and regional volume were obtained by systematic-random sampling in the right SN, including the SN pars compacta (SNpc) and the SN pars reticulata (SNpr) from Bregma level -4.36 mm through -6.72 mm, according to the Paxinos and Watson rat brain atlas. To allow for a mathematical estimate of the total population of neurons, neurons in a defined area of each tissue section, through a known depth of the tissue section, in a known fraction of tissue sections, were counted as described below. Counting the number of systematically random points falling within a region of interest provided an estimate of area, which can be extrapolated to regional volume based on the Cavalieri principle (see below). Measuring the diameter of a neuronal nucleus on a length cubed ruler at a random orientation allowed for a mathematical estimate of nuclear volume (see below).

Fractionator: Neuronal number estimate—The SN was identified based on morphological and histological boundaries: the lateral and ventral extent by the cerebral peduncle and the medial margin by the ventral tegmental area and medial lemniscus. Every second section containing the SN was selected to obtain a sample in a systematic uniform random manner (section-sampling fraction; $ssf = 0.5$), yielding 10–19 sections per brain. In the sampled sections, an optical dissector counting frame was employed for counting and measuring neuronal nuclei at predetermined regular x,y intervals (Gundersen et al., 1988). Inclusion and exclusion counting criteria were followed by a blinded observer who recorded counts only when a single neuronal nucleolus was brought into focus within the dissector frame (Sterio, 1984). Healthy neurons were distinguished from other objects such as astrocytes and microglia based on the presence of a single, readily distinguishable nucleus within the cell in question, in accordance with criteria previously used in stereological studies to identify neurons (Grady et al., 2003; Lifshitz et al., 2007; Witgen et al., 2005). To be counted, neurons possessed a darkly stained nucleolus and unstained nucleus within central region of a stained soma; the plasma membrane was visible and intact. Unhealthy neurons (dystrophic or multiple nuclei and/or inconsistent nuclear membranes) were not quantified. Because the area (a) of the counting frame ($2,500 \mu\text{m}^2$) was known relative to the regular stage-stepping intervals over the section ($250 \mu\text{m} \times 250 \mu\text{m}$), one can calculate the area sampling fraction (asf) = $a(\text{frame}) / a(x,y \text{ step})$ as 0.04. The height (h ; $25\text{--}29 \mu\text{m}$) of the optical dissector was equivalent to the thickness of the section (t ; $27.3 \pm 0.7 \mu\text{m}$). With these parameters, the estimated number of neurons (N) followed from the formula $N = \Sigma Q^- \cdot t/h \cdot 1/asf \cdot 1/ssf$, where ΣQ^- was the number of neurons counted. The average number of neurons counted was 266 ± 9 , 229 ± 45 and 149 ± 25 for uninjured sham, 7 day and 28 day mFPI animals, respectively.

To analyze the sampling scheme reliability, for every animal, coefficient of error (CE) was calculated using Matheron's quadratic approximation (Gundersen and Jensen, 1987) and by considering the “nugget effect” (West et al., 1996) to reflect the variance introduced by the sampling of tissue sections. The coefficients of error were 0.07, 0.08 and 0.10 for uninjured sham, 7 day and 28 day mFPI animals, respectively. All sampling was conducted using an Olympus BH-2 microscope with an ASI automatic stage (Olympus, USA), using a $100\times$, 1.3 numerical aperture oil immersion objective (Olympus, Japan). A mounted video camera (QImaging, BC, Canada), and microcator (Heidenhain, Deerfield, IL) were used in conjunction with Bioquant Life Science Image Analysis software package (Bioquant, Nashville, TN), which included stereology and topography plugins.

Cavalieri principle: Neuronal density estimate—Estimates of regional volume were obtained by the Cavalieri method, based on systematic random point counting (Lifshitz et al., 2007). The volume of the SN was estimated by counting the number of unique points

along a systematic random grid ($250\ \mu\text{m} \times 250\ \mu\text{m}$) across the outlined region that fell within the region of interest. Each included point represents a known area, such that the sum of the number of points lying within the region multiplied by the corresponding area ($62,500\ \mu\text{m}^2$) multiplied by the represented thickness ($30\ \mu\text{m} \times 2$ sections) yields an estimate of the SN volume for each animal. Neuronal density was calculated as the number of neurons per cubic micron for each animal.

Vertical nucleator: Neuronal nuclear volume estimate of atrophy—Unbiased object volume (μm^3) can be estimated using the vertical nucleator stereological probe. As employed in the present communication, the vertical nucleator provided assumption-free estimates of mean neuronal nuclear volume in systematically sampled neurons within the SN (Gundersen et al., 1988; Gundersen, 1988; Lifshitz et al., 2007; Lifshitz and Lisembee, 2011). When the neuronal nucleolus was in focus, the nucleator was applied. Two randomly oriented, perpendicular length cubed rulers (L^3) extend from a central point in the nucleolus and the intersections of the rulers with the nuclear boundary were marked. Measurements of nuclear volume, rather than somatic volume, provided clear and objective boundaries of the nuclear membrane surrounding the unstained nucleus. The BioQuant software package calculated the neuronal volume based on the length cubed rulers. The nucleator was applied to an average of 214 ± 21 neurons per animal (range: 93–327).

Whole tissue content of dopamine and dopamine metabolites

Animals were decapitated after CO_2 asphyxiation and their brains rapidly removed. The brains were placed in an ice-cold rat brain matrix and sliced into 1 mm sections. The SN (approximately -4.8 – -5.8 mm posterior to Bregma) and dorsal and ventral striatum (approximately 0.0 – $+1.0$ mm anterior to Bregma) were dissected bilaterally. Tissue was weighed in pre-weighed microfuge tubes, frozen on dry ice and stored at -70°C (Hudson et al., 1995). Dissected tissue was prepared for analysis by addition of an internal standard, dihydroxybenzylamine (DHBA), to provide an index of recovery. Tissue was then sonicated in cold citrate-acetate buffer mobile phase (pH 4.1) and centrifuged at $16,000 \times g$ for 10 min. Dopamine and its metabolites 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) in the $50\ \mu\text{L}$ supernatant were separated by reverse-phase high-pressure liquid chromatography with electrochemical detection (HPLC-EC). Separation of the analytes was achieved by a C18 column ($4.6\ \text{mm} \times 75\ \text{mm}$, $3\ \mu\text{m}$ particle size, Shiseido CapCell Pak UG120, Shiseido Co., LTD., Tokyo, Japan). The mobile phase flow rate was $2.0\ \text{mL/min}$. Detection was carried out using a dual channel coulometric detector (ESA model 5011A dual analytical cell) with potentials of $E_1 = +350\ \text{mV}$ and $E_2 = -250\ \text{mV}$. The peak area and the retention times of the standards were used to quantify dopamine and dopamine metabolite levels in the tissue (nanograms of dopamine or dopamine metabolite per gram of wet tissue weight) with hemispheres averaged together for each animal. A more detailed review of this protocol can be found in the original work (Salvatore et al., 2005).

Tissue preparation and immunohistochemistry

At designated time points post-injury, animals were euthanized by an overdose of sodium pentobarbital ($150\ \text{mg/kg}$, i.p.) and transcardially perfused with 4% paraformaldehyde in phosphate-buffered saline. Brains were removed, cryo-preserved in 30% sucrose and sectioned in the coronal plane at $30\ \mu\text{m}$ on a cryostat. Sets of tissue sections were immunostained to identify injury-induced changes in the neurovascular unit. Briefly, endogenous peroxidase activity was quenched with 0.9% H_2O_2 in EtOH and tris-buffered saline (TBS) for 20 min. After antigen retrieval (5% citric acid, 30 min, 45°C), sections were pre-incubated in 10% normal horse serum (NHS) with 0.2% Triton X-100 in TBS (60 min). Sections were incubated overnight with primary antibody in 1% NHS in TBS. Primary antibodies included tyrosine hydroxylase (1:1,000, AB152, rabbit anti-rat, Millipore) and

immunoglobulin G (biotinylated IgG; 1:5,000, BA-9400, goat anti-rat, Vector Labs). The following day, sections for tyrosine hydroxylase were incubated in biotinylated goat anti-rabbit IgG secondary antibody (60 min, 1:200; Vector Labs) in 1% normal goat serum (NGS) in phosphate-buffered saline (PBS). Immunostaining was visualized using avidin:biotin enzyme complex (Vectastain ABC Standard Elite Kit; Vector Labs) for 1 hour followed by 0.04% diaminobenzidine and 0.006% H₂O₂ in 0.1M TBS for 10–20 minutes. Sections were dehydrated and coverslipped. Additional sections processed without primary antibody served as a negative control. Images were captured using an Olympus AX80 microscope equipped with an integrated digital camera and image capture software. Final publication micrographs were adjusted to utilize the maximum range of grayscale levels (Adobe Photoshop CS2). Immunostaining for ionized calcium binding adaptor molecule 1 (Iba-1) was performed by Neuroscience Associates (Knoxville, TN) concurrently with the de Olmos silver stain, according to standard protocols. Three Iba-1 stained tissue sections per animal (–4.6, –5.2, –6.0) were examined by light microscopy for morphological change in microglia phenotype.

Estimate of TH-Positive Neuron Number

Bioquant Image Analysis software (Bioquant Image Analysis, Nashville, TN) was used to estimate TH-positive cell number in the substantia nigra using the fractionator method, as previously described (Liu et al., 2008; Liu et al., 2010). For these estimates, a computer-assisted image analysis system consisted of a Zeiss Axioskop2Plus photomicroscope equipped with a MS-2000 (Applied Scientific Instrumentation, Eugene, OR) computer-controlled motorized stage, a 3CCD color video camera (Sony, Japan), a DELL GX260 workstation, and Bioquant stereology toolkit. Sections in a series composed of every sixth section of the substantia nigra (total 6 sections per brain) were immunostained for TH and cell numbers were counted at a 40× objective following a systematically random sampling scheme. The disector height was 10 µm. Both grid cell area and counting frame area were set at 120 µm × 120 µm. Brains were fixed using the same protocol, tissues were cryosectioned with a cryostat microtome, the immunostaining steps were performed on all of the tissue sections at once, and cell counting was conducted in a blinded fashion. Statistical differences between uninjured sham and brain-injured animals were tested using an unpaired, two-tail t-test.

Aminocupric analysis of neurodegeneration

Neurodegeneration was examined using the de Olmos aminocupric silver histochemical technique as previously described (de Olmos et al., 1994; Hall et al., 2008; Switzer, 2000). At designated times, sham or mFPI rats were overdosed with sodium pentobarbital (200 mg/kg i.p) and transcardially perfused with 0.9% sodium chloride, followed by a fixative solution containing 4% paraformaldehyde. Following decapitation, the heads were stored in a fixative solution containing 15% sucrose for 24 h, after which the brains were removed, placed in fresh fixative, and shipped for histological processing to Neuroscience Associates Inc. (Knoxville, TN). The rat brains were embedded into a single gelatin block (Multiblock® Technology; Neuroscience Associates). Individual cryosections containing all the rat brains were mounted and stained with the de Olmos silver staining methods according to proprietary protocols (Neuroscience Associates) to reveal argyrophillic tissue, including neurons and neuronal processes, counterstained with Neutral Red, and then coverslipped. The stained sections were analyzed in our laboratory.

Quantification of the silver precipitate in the SN was conducted by calculating the percentage of silver-positive pixels within strict structural landmarks (described above for stereological quantification) from grayscale digital photomicrographs, comparable to the pixel quantification as previously described (Hall and Lifshitz, 2010). Briefly, the grayscale

pixel distribution was digitally thresholded to separate positive stained pixels from unstained pixels (BioQuant, BioQuant Image Analysis Corporation) by a single un-biased observer. The thresholded image was then segmented into white and black pixels, indicating positive and negative staining, respectively. Four to six sections (Bregma level -4.36 mm through -6.72 mm) per animal containing the SN were quantified in triplicate to calculate the silver staining as percent of positive silver stained pixels (white pixels) in the region of interest (white + black pixels).

Quantitative real-time PCR (qRT-PCR)

Additional animals were generated for quantitative real-time polymerase chain reaction (qRT-PCR), which has the sensitivity to detect the expression of low abundance mRNA transcripts in small tissue samples of individual animals. Sham, 7 day and 28 day mFPI rats were perfused with ice-cold saline, and tissue samples from the SN were obtained by biopsy from 2 mm coronal sections. The mRNA content was stabilized (RNAlater, Qiagen Corp) and stored frozen. Isolated mRNA (RNeasy, Qiagen Corp) was quantified (NanoDrop ND-1000 spectrophotometer), converted to complementary DNA (cDNA; High Capacity cDNA Archive Kit, Applied Biosystems Inc.) and then used as a template (500–2500 pg) based on original RNA concentrations for commercially-available gene expression assays, according to manufacturer's protocols. Injury-related gene expression responses were quantified in triplicate (StepONE, Applied Biosystems) using the manufacturer's protocols. The Applied Biosystems TaqMan® Gene Expression Assays are optimized to run under universal thermal cycling conditions, with amplification efficiencies of 100%. Assays were performed for the following genes: tyrosine hydroxylase (Rn00562500_m1), dopamine transporter (DAT; Rn00562224_m1), cluster of differentiation molecule 45 (CD45: RN00709901_m1) and translocator protein 18 kDa (TSPO; Rn00560892_m1). Within each animal, relative gene expression was normalized to the 18s rRNA endogenous control (part number 4352930E) and the average threshold cycle in the sham group using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001), which relates gene expression to the PCR cycle number at which the fluorescence signals exceed a threshold above baseline.

Radioligand binding

Uninjured sham and mFPI animals were euthanized by decapitation, and then the brains were removed and frozen in an isopentane-dry ice slurry. The brains were stored at -80°C until further processing. Brains were sliced on a cryostat (Lecia CM1850, Nussloch, Germany) into a series of 16- μm -thick sections. The dopamine transporter (DAT) and translocator protein 18 kDa (TSPO) receptor densities were measured using [^{125}I]-RTI-55 and [^3H]-PK-11195 autoradiography, respectively, as previously described (Kelso et al., 2009; Little et al., 1998). Ligand concentrations of 1 nM [^{125}I]-RTI-55 specific activity 2200 Ci/mmol and [^3H]-PK-11195 specific activity 85.5 Ci/mmol (Perkin-Elmer Life Sciences, Boston, MA, USA) were used for incubation. RayMax Beta High Performance Autoradiography Film (ICN Biomedicals, Aurora, OH) was used to visualize ligand binding. Radioactive rat brain tissue standards were included in each film cassette. Exposure time was optimized for each ligand (8 days for [^{125}I]-RTI-55; 29 days for [^3H]-PK-11195). All films were processed using Kodak GBX developer. Binding data were analyzed using NIH image v1.59 on a Power Macintosh connected to a Sony XC-77 CCD camera via a Scion LG-3 frame-grabber. The SN was outlined manually to include the SNpc and the SNpr. Mean staining density was calculated for each individual section in triplicate, across 18 to 24 coronal sections (approximately Bregma level -4.36 mm through -6.72 mm) to obtain an overall mean binding density for each animal.

Statistical analysis

Data are presented as mean $\pm$ SEM for all studies. Image analysis, radioligand binding and relative gene expression studies were analyzed by one-way ANOVA followed by a Neumann-Keuls post-test, with significance set at $p < 0.05$.

Results

Neurons

This section reports neuronal damage recorded in the SN in somal, axonal and synaptic compartments after moderate diffuse brain injury.

Neuronal cell loss is evident without atrophy of surviving cells at 28 days post-injury

—Across the time course studied, neuronal cell loss, without atrophy, was evident in SN (compacta and reticulata) after experimental diffuse TBI. Using stereological techniques, we quantified neuronal number, density, and nuclear volume for uninjured sham ($n = 5$), 7 day ($n = 5$) and 28 day ($n = 5$) mFPI rats. Giemsa-stained coronal sections of the SN of sham (Figure 1A) and 28 day (Figure 1B) rats showed decreased intensity and distribution of cellular staining, based on microscopic evaluation. Stereological estimates of neuronal number using the optical fractionator technique indicated a significant reduction in neuronal number between groups ($F(2,12) = 3.77$; $p = 0.0493$), revealing a significant 44% neuronal loss in the substantia nigra (pars compacta and reticulata combined) by 28 days after moderate midline FPI ($9,871 \pm 1580$) compared to uninjured sham ($17,251 \pm 584$; Figure 1C). Normalizing to regional volume confirmed a significant reduction in neuronal density at 28 days post-injury ($32,242 \pm 4,429$), compared to both sham ($46,913 \pm 2,805$) and 7 days post-injury ($48,715 \pm 3,697$; $F(2,12) = 5.95$; $p = 0.0160$; Figure 1D). As an indication of neuronal atrophy, estimates of the mean neuronal nuclear volume of the remaining neurons were similar across groups (Sham: 251.2 ± 10.1 ; 7d FPI: 246.9 ± 9.1 ; 28d FPI: 281.0 ± 17.3 ; $F(2,12) = 0.407$; $p = 0.6748$; Figure 1E). Thus, it is apparent that by 28 days post-single diffuse brain injury of moderate severity resulted in neuronal cell loss, but not atrophy, as quantified by cell number and density.

Whole tissue dopamine metabolite levels suggest compensation for neuronal loss

—The stereological findings indicated a loss of dopaminergic neurons from the SN. Using HPLC-EC to assess dopamine tissue levels in the SN, dorsal striatum and ventral striatum, we hypothesized injury-induced decreases in line with the loss of healthy neurons after brain injury. However, total tissue dopamine levels showed non-significant increases after brain injury ($n=5$) compared to uninjured control ($n=4$) of 7.1% in the SN (Sham: 528 ± 85 ; 28d FPI: 566 ± 69 ; $t_7 = 0.3467$, $p = 0.7390$; Figure 2A), 14.0% in the dorsal striatum ($t_7 = 0.4641$, $p = 0.6566$) and 9.0% in the ventral striatum ($t_7 = 0.2566$, $p = 0.8049$). Dopamine (DA) metabolites include 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA), and their quantification revealed an altered neurotransmitter turnover. The injured SN showed a non-significant 26.5% reduction in DOPAC (Sham: 292 ± 60 ; 28d FPI: 215 ± 36 ; $t_7 = 1.153$, $p = 0.2869$; Figure 2B) and a significant 44.6% reduction in HVA (Sham: 368 ± 22 ; 28d FPI: 204 ± 30 ; $t_7 = 4.219$, $p = 0.0039$; Figure 2C) compared to these metabolites in sham. The DA turnover ratio (DOPAC + HVA / DA) has been used to represent DA synthesis, release and metabolic pathways previously (Thiffault et al., 2000). Diffuse brain injury impaired DA signaling in the SN, as indicated by the significant reduction in the DA turnover ratio (Sham: 1.30 ± 0.13 ; 28d FPI: 0.76 ± 0.09 ; $t_7 = 3.509$, $p = 0.0099$; Figure 2D). For the striatum, tissue DOPAC levels did not change significantly after brain injury, showing decreases from uninjured control of 38.6% in the dorsal striatum ($t_7 = 2.646$, $p = 0.0773$) and 33.0% in the ventral striatum ($t_7 = 1.563$, $p = 0.1930$). Tissue HVA levels did not change significantly after brain injury, showing increases of 2.9% in the mFPI

dorsal striatum ($t_7 = 0.1381$, $p = 0.8968$) and decreases of 9.9% in the ventral striatum ($t_7 = 0.4848$, $p = 0.6450$) from uninjured control. It follows that no significant differences were detected in the DA turnover ratio in the dorsal striatum ($t_7 = 2.119$, $p = 0.1014$) and ventral striatum ($t_7 = 1.618$, $p = 0.1665$) in comparison to sham controls.

To evaluate whether injury effects DA synthesis, we measured tyrosine hydroxylase (TH) protein localization and gene expression. No statistically significant difference in TH-positive pixels within the SN was identified between sham (Figure 3A; $n = 3$) and 28 day (Figure 3B; $n = 3$) mFPI animals ($t_4 = 1.599$, $p = 0.1851$; Figure 3C). This result appeared antithetical compared to the loss of cells measured by stereology. The estimated neuronal dropout may not represent an entirely dopaminergic population, unless the remaining healthy neurons produce more TH through additional synthetic machinery. As measured by qRT-PCR, relative TH mRNA levels, an indirect assessment of TH synthesis machinery, showed a non-significant increase in gene expression between uninjured sham ($n = 4$) and 28 day ($n = 4$) mFPI animals ($t_6 = 1.349$, $p = 0.2260$; Figure 3D) compared to an endogenous control (18s rRNA). Further, stereological estimates showed a significant 25% reduction in the number of TH-positive neurons in the SN by 28 days after diffuse brain injury ($22,345 \pm 1852$; $n = 4$) compared to sham ($29,980 \pm 1500$; $n = 3$; $t_5 = 3.023$, $p = 0.0293$), suggesting partial loss and commensurate compensation for overall reductions in SN dopamine content.

Injury-induced progressive neurodegeneration across the SN—The de Olmos silver stain technique does not specifically identify the manner of axonal pathology, but serves as a marker of general neuronal degeneration and reorganization, which may be concomitant with general neuronal loss. Silver-stained sections of SN from sham ($n = 3$; Figure 4A), 7 day ($n = 3$; Figure 4B) and 28 day ($n = 3$; Figure 4C) diffuse mFPI rats showed striking increases in neurodegeneration as indicated by widespread argyrophilia. Pixel quantification of silver stain in the SN confirmed significant neurodegeneration at 7 days ($21.2\% \pm 1.4$) and further significant pathology at 28 days post-injury ($29.2\% \pm 2.8$) compared to uninjured sham ($1.0\% \pm 1.0$; $F(2,6) = 59.814$, $p < 0.0001$; Figure 4D). Neuronal tracts within the SN exhibited progressive neurodegeneration after diffuse brain injury.

Dopamine transport remains unaffected by diffuse TBI—The dopamine transporter (DAT) is localized peri-synaptically on dopaminergic neurons and can be visualized and quantified using the radiolabeled ligand [125 I]-RTI-55. DAT protein expression in the SN was quantified in sham ($n = 6$; Figure 5A), 7 day ($n = 5$; Figure 5B) and 28 day ($n = 5$; Figure 5C) mFPI rats. Density quantification of [125 I]-RTI-55 radioligand binding indicated no significant changes in DAT expression over time post-injury compared to sham ($F(2,13) = 1.353$, $p = 0.2925$; Figure 5D). To assess whether the absence of a change in protein expression was due to increased gene expression despite cellular loss, we quantified relative DAT mRNA levels. However, no significant differences were observed between sham ($n = 4$), 7 day ($n = 5$) and 28 day ($n = 5$) mFPI rats ($F(2,11) = 1.881$, $p = 0.1983$; Figure 5E). Neither protein nor gene expression for DAT were altered as a function of time after a single moderate diffuse brain injury.

Vasculature

Multi-focal sites of blood brain barrier compromise acutely after diffuse brain injury—The mechanical forces that initiate neurodegeneration could similarly result in vascular permeability that contributes to the observed SN pathology. To assess blood brain barrier (BBB) permeability, immunohistochemical assessment was undertaken for parenchymal localization of the large blood-borne immunoglobulin G (IgG) molecule (Habgood et al., 2007; Povlishock et al., 1978; Rinder and Olsson, 1968; Tanno et al., 1992).

Uninjured sham brains did not show parenchymal localization of IgG ($n = 3$; Figure 6A). In line with previous reports of the rapid re-sealing of the compromised BBB (Cortez et al., 1989; Huh et al., 2008), IgG extravasation was identified across the mFPI SN at 1 day ($n = 3$; Figure 6B) and 2 days ($n = 3$; Figure 6C) post-injury, in at least two sections per animal. No appreciable immunostaining for IgG could be detected at 7 days and 28 days post-injury in any animals (data not shown).

Microglia

Diffuse brain injury results in microglial activation and macrophage infiltration in the SN—Ionized calcium binding adaptor molecule (Iba-1)

immunohistochemistry was used to identify morphological change in microglia in response to insult. The Iba-1 staining showed highly ramified microglia with spherical cell bodies in sham animals (Figure 7A) that became less ramified with swollen cell bodies at 2 days post-injury (Figure 7B). Over the 7 day (Figure 7C) and 28 day (Figure 7D) post-injury time course, microglial morphology resumed the higher ramified phenotype, but to a lesser extent than in sham animals with an apparent lower cell density.

The translocator protein 18kDa (TSPO; cholesterol transporter located on the outer mitochondrial membrane of glia (Chen and Guilarte, 2008)) is expressed by microglia activated in response to injury or neurodegenerative conditions (Chen and Guilarte, 2008). The isoquinoline carboxamide [^3H]-PK-11195 binds with high affinity to the TSPO receptor, indicating regions of inflammation, either through increased expression or infiltration. The selectivity and sensitivity of this technique was evident in the absence of specific binding in sham animals outside of the ependymal cells of the choroid plexus lining the ventricles ($n = 6$; Figure 8A). The binding of [^3H]-PK-11195 in tissue sections from diffuse mFPI animals indicated a global glial response within 2 days ($n = 4$; Figure 8B) that remained evident through 7 days ($n = 5$; Figure 8C) and 28 days ($n = 5$; Figure 8D) post-injury. Regional binding affinity highlights the injury-induced specificity of neuroinflammation in the SN. Other brain regions (e.g. thalamus, hippocampus, cortex) will be reported in separate publications; the striatum showed no appreciable binding. Image density measurements of [^3H]-PK-11195 radioligand binding indicated a significant increase at 7 days post-injury compared to sham ($F(3,16) = 3.563$, $p = 0.0380$; Figure 8E), and non-significant increases at 2 days and 28 days post-injury. Using qRT-PCR, the relative TSPO gene expression in the SN increased over time post-injury, becoming significant at 28 days ($n = 6$) compared to sham ($n = 6$) and the 7 day time point ($n = 6$; $F(2,15) = 6.635$, $p = 0.0086$; Figure 8F). To demonstrate the persistent inflammatory response in the mFPI SN, gene expression for the pan-leukocyte marker CD45 (cluster of differentiation protein 45, alternately known as protein tyrosine phosphatase receptor type C) was quantified as an indicator of inflammation by infiltrating leukocytes (Dick et al., 2001). Gene expression of CD45 over the post-injury time course revealed significant CD45 expression at 28 days ($n = 6$) compared to sham ($n = 6$) and the 7 day time point ($n = 6$; $F(2,15) = 9.785$, $p = 0.0019$; Figure 8F). These data support the conclusion that neuroinflammatory processes persist in the diffuse mFPI SN.

Discussion

Recent post-mortem studies of American football players have found pallor or paleness of the SN (McKee et al., 2009; Omalu et al., 2005; Omalu et al., 2006; Omalu et al., 2010), prompting us to explore neurovascular pathology in the SN after a single experimental diffuse brain injury. The present study using the mFPI model demonstrates significant decreases in total and TH-positive neuronal cell number and total neuronal density by 28 days post-injury, without change in neuronal nuclear volume. Moreover, DA regulation is

altered as indicated by reductions in DA metabolism but not necessarily its synthesis. Histological hallmarks of neurodegeneration (argyrophilia) persist throughout the observed time course, without appreciable changes in DA transport. In addition, the mechanical forces of the injury disrupt the blood brain barrier, which may initiate the enduring neuroinflammation of the SN and thereby contribute to early and late stages of clean-up and repair. We propose that compensatory mechanisms to maintain dopamine content mask injury-induced effects on nuclear volume, DA synthesis and transport. Hence, a single moderate diffuse TBI leads to reductions in neuron number, DA turnover, persistent neurodegeneration and inflammation within the SN, which may initiate a susceptibility to age-related cumulative degeneration or repeated trauma events (Litvan et al., 2007).

Selective vulnerabilities following experimental TBI

Primary neuronal loss from the SN after diffuse brain injury was the predominant histopathology, which resembles the beginning of parkinsonism pathology, particularly since significant loss of TH-positive neurons was observed. Neuronal atrophy, which has become a histopathological hallmark of diffuse brain injury (Lifshitz et al., 2007; Maxwell et al., 2004; Maxwell et al., 2010), does not necessarily extend into the SN. Previous lateral FPI studies have noted (but not quantified) histopathological damage in the SN in the ipsilateral hemisphere as early as 10 minutes, extending up to 7 days post-injury (Hicks et al., 1996). Moreover, the cerebral metabolic rate of glucose following similar brain injuries was not reported to change in a time course up to 10 days post-injury (Hovda et al., 1991; Yoshino et al., 1991). In our model, neuropathology is compounded by significant neuronal debris and chronic neuroinflammation (see below). Injury-induced argyrophilia may represent the poorly myelinated axons of DA containing neurons of the SN (Braak et al., 2006), making them more vulnerable to axonal damage (Reeves et al., 2005). Alternatively, axonal fibers passing in the vicinity of the SN and the cerebral peduncle may contribute to the observed neurodegeneration. Future studies should explore more specific neuropathology, including α -synuclein, TDP-43, ubiquitin and tau pathologies in axons, dendrites and synapses (Jellinger, 2009) in experimental brain injury models. Despite the aforementioned neuropathology, the SN retains capacity to synthesize DA, but not necessarily to metabolize it, likely through compensatory mechanisms in surviving TH-positive neurons, given that only a single moderate brain injury was induced.

The possibility remains for a predilection to damage of dopaminergic neurons of the SNpc due to inherent physiological characteristics. Nigral neurons have an inherently high baseline activity with an irregularly elevated metabolism in the absence of synaptic input (Nedergaard et al., 1993). The increased frequency of opening of L-type calcium channels at baseline and during stimulation leads to increased cytosolic calcium levels, resulting in elevated mitochondrial oxidative stress and vulnerability to toxins (Lotharius et al., 1999; Pedrosa and Soares-da-Silva, 2002; Surmeier et al., 2010a, 2010b). DA itself could be a root cause, whereby DA oxidation or its metabolites leads to cellular damage (Greenamyre and Hastings, 2004; Sulzer, 2007), particularly given the observed reductions in DA metabolism. Not only are dopaminergic neurons more vulnerable than neighboring neuronal populations, specializations within the SNpc have been reported to be more at risk than the SNpr in Parkinson's disease in humans (Simunovic et al., 2009). Additionally, the lateral regions of the SN have been reported to be more vulnerable acutely than the more medial regions (Duke et al., 2007). A 25% loss of dopaminergic neurons from a single diffuse brain injury suggests a limited capacity to sustain further insults or injury. Mechanical trauma and its secondary cascades would place additional strains on this vulnerable system. As the metabolic or functional challenges on the system accumulate, progressive degeneration evolves as reported here. This vulnerability to subsequent insults is highlighted by the recent report in which acute exposure to the herbicide and dopaminergic toxin paraquat augmented

total and dopaminergic substantia nigra neuronal death after TBI in the rat (Hutson et al., 2011). The response to repeated diffuse brain injury and longer time points remain to be evaluated.

Compensation for neuronal loss

Reductions in DA release and metabolism across the nigrostriatal pathway were hypothesized to be concomitant with neuronal loss in the SN. However, we report unchanged tissue DA levels in the SN and striatum, in addition to non-significant changes in TH and DAT protein levels and gene expression in the SN. Yet, up to 25% of TH-positive neurons were lost by 28 days post-injury. Total tissue dopaminergic synthesis may be preserved by the upregulation of gene expression and protein translation within surviving healthy neurons; however the techniques employed cannot determine the contribution of individual neurons to the whole tissue analysis. Compensatory mechanisms (e.g. increased TH activity or DA vesicular release) could maintain DA levels, and remain to be explored. The possibility remains for the sprouting of DA terminals in surviving TH-positive neurons, which could explain the maintenance of DAT levels in brain-injured tissue. Similar compensatory mechanisms are predicted to occur in neurotoxic models of parkinsonism by partial lesion of the SN, where parkinsonism-like symptoms are detectable only when markers of dopaminergic neurons in the SN fall below 20–40% of normal values (Anastasia et al., 2009; Breit et al., 2007; Dauer and Przedborski, 2003; Di Monte et al., 2000), resulting in failure of compensatory mechanisms brain-wide. These studies support the possibility for compensatory processes within the SN to maintain DA synthesis up to one month after a single diffuse brain injury. Compensation for subsequent losses from repeated or more severe injuries may not be possible (Litvan et al., 2007).

Significant reductions in DA metabolites and the DA turnover ratio imply injury-related reductions in DA release and/or metabolism, which may represent partial compensation for the injury-induced neuronal loss and degeneration. Across species, partial lesions of the striatum decrease DA relative to HVA (Bernheimer and Hornykiewicz, 1965; Hornykiewicz, 1966; Zigmond, 1997), indicating increased DA metabolism. In normal aging, levels of HVA and DOPAC are decreased across the rodent basal ganglia (Hebert and Gerhardt, 1998; Stanford et al., 2003), indicating an age-related deterioration of the ability to metabolize DA (Hebert and Gerhardt, 1998). Whether by injury or aging, changes in monoamine oxidase activity could contribute to compensatory processes to mask SN degeneration (Kopin, 1994), but have yet to be explored in experimental brain injury. Since DA levels may be partially maintained by reductions in metabolism, no changes in DAT transporter were expected, as reported. Alternatively, neuroplastic or homeostatic changes in DA terminals, whether by sprouting or increasing in size, could bolster markers of DA function. Thus, decreases in DA metabolism after brain injury could compensate for injury-related neuropathology. The duration for which these compensatory processes can function in the injured brain before exhaustion remains to be determined.

Persistent microglial activation across acute and chronic time points

Vascular compromise within the first days post-injury as indicated by parenchymal localization of IgG and serum albumin shows that mechanical forces may contribute to neurovascular injury in the SN (Kelley et al., 2007). Ruptured blood vessels and damaged neurons release a host of factors that can activate microglia (Streit et al., 1999). Activated microglia were identified by altered morphology over the 28 day time course and verified by TSPO and CD45 expression, in accordance with cortical and thalamic regions (Kelley et al., 2007). Whether the inflammatory response arises from cytokine signaling of neuronal or vascular origin remains to be determined, but confirms pathological cascades within the SN.

Radioligand binding of [^3H]-PK-11195 to TSPO verified the time course and extent of microglial activation in the mFPI SN as seen morphologically with Iba-1 staining. TSPO is normally expressed at low levels throughout the brain, becoming up-regulated in response to brain injury (Chen and Guilarte, 2008; Guilarte et al., 1995), and particularly in the basal ganglia within 5 days after neurotoxic insult (Kuhlmann and Guilarte, 1997). Acute post-injury increases in [^3H]-PK-11195 binding have been associated with gliosis and classical microglial activation, whereas persistent changes in TSPO expression have been linked to remodeling and repair processes (Chen and Guilarte, 2008). Experimental brain injury is associated with an acute phase secretion of pro-inflammatory cytokines (Wang and Shuaib, 2002). Upon injury, microglia switch from an 'inactive' sensing of the microenvironment to an 'active' phagocytic form that can engulf axonal and neuronal debris generated by the injury (Reichert and Rotshenker, 1996). At more chronic time points, reactive glia can protect and repair injured tissue by secreting trophic growth factors, such as glial cell line-derived neurotrophic factor (GDNF) (Kreutzberg, 1996; Neumann et al., 2006; Thored et al., 2009). GDNF infusion can protect dopaminergic circuits (Gash et al., 1998; Stanford et al., 2003).

After a single brain injury, microglia remain disturbed up to a month, if not longer, implying that the neurovascular unit may be susceptible to further damage. Persistent microglial activation could go on to affect originally unaffected neurons, contributing to the continued atrophy reported in the development of parkinsonism (Przedborski, 2010). In this way, a mechanically induced injury may act as a priming event to activate microglia (Long-Smith et al., 2009). Ensuing head injuries or environmental exposure may promote the onset, magnitude or constellation of parkinsonian symptoms (Litvan et al., 2007; Surmeier et al., 2010b).

Symptomatic implications of neurodegeneration of the SN

The present study documents neurovascular damage in the SN following a single moderate diffuse brain injury. This level of injury may be below thresholds detected by behavioral measures, particularly because the SN may compensate for the observed damage. Unilateral therapeutic interventions to spare the SN or sub-threshold neurotoxic lesions after mFPI may replicate behavioral features of parkinsonism following unilateral neurotoxic lesions (Mendez and Finn, 1975; Minnich et al., 2010; Mokry, 1995; Nakajima et al., 2010). Repeated trauma, normal aging or other life experiences may become significant when compounded with the incremental loss of neurons and ongoing neurodegeneration across more chronic time points (Litvan et al., 2007). At a particular threshold, the remaining neurons may fail to compensate for the injury-induced alterations in dopaminergic signaling. This observed pathology could develop into clinical symptomatology in sportspeople who suffer multiple concussions, and over longer time periods develop parkinsonian symptoms indicative of neuronal loss and subsequent signaling deficits in the SN (Casson et al., 2006; Omalu et al., 2005; Omalu et al., 2006; Omalu et al., 2010). Single or repeated head injury in young adult males may be a principal factor responsible for the prevalence of parkinsonism amongst males (Langlois et al., 2004; Litvan et al., 2007). These clinically relevant scenarios remain to be explored in animal models.

Concluding Remarks

These data comprise the first comprehensive study of the time course of neurodegeneration across the neurovascular unit of the SN following experimental diffuse brain injury. The profound loss of neurons highlights the vulnerability of the SN, whilst the maintenance of dopaminergic signaling insinuates an ability of the remaining cells to compensate for the sub-threshold damage. Whether this compensation can be maintained or endure, remains to be seen. Investigations over a protracted time course may identify a post-injury time point at

which compensatory processes fail and deficits emerge, perhaps due to normal aging. Different injury severity levels or repeated injury study designs could address these further clinically relevant issues associated with injury-induced degeneration of the SN. From a clinical perspective, the host of changes in the SN following a single moderate diffuse TBI highlights the hidden dangers from which sportspeople are at risk following multiple concussive or sub-concussive injuries; the ensuing symptomatology could manifest chronically past the acute signs of concussion.

Acknowledgments

With generous thanks to Amanda Lisembee, Deanne Hopkins, Kelley Hall and Stewart Surgener without whose technical expertise and insightful support this study would not have been possible. Supported, in part, by University of Kentucky College of Medicine, NIH NINDS R01 NS065052, NIH NIA T32 AG000242, NIH NINDS F31 NS067899, Kentucky Spinal Cord and Head Injury Research Trust (KSCHIRT) 7–11, NIH P50 NS039787 and NIH NINDS P30 NS051220.

References

1. Anastasia A, Torre L, de Erausquin GA, Masco DH. Enriched environment protects the nigrostriatal dopaminergic system and induces astroglial reaction in the 6-OHDA rat model of Parkinson's disease. *J.Neurochem.* 2009; 109:755–765. [PubMed: 19245661]
2. Bernheimer H, Birkmayer W, Hornykiewicz O, Jellinger K, Seitelberger F. Brain dopamine and the syndromes of Parkinson and Huntington. Clinical, morphological and neurochemical correlations. *J Neurol Sci.* 1973; 20:415–455. [PubMed: 4272516]
3. Bernheimer H, Hornykiewicz O. Decreased homovanillic acid concentration in the brain in parkinsonian subjects as an expression of a disorder of central dopamine metabolism. *Klin.Wochenschr.* 1965; 43:711–715. [PubMed: 5826380]
4. Bove J, Prou D, Perier C, Przedborski S. Toxin-induced models of Parkinson's disease. *NeuroRx.* 2005; 2:484–494. [PubMed: 16389312]
5. Braak H, Bohl JR, Muller CM, Rub U, de Vos RA, Del TK. Stanley Fahn Lecture 2005: The staging procedure for the inclusion body pathology associated with sporadic Parkinson's disease reconsidered. *Mov Disord.* 2006; 21:2042–2051. [PubMed: 17078043]
6. Breit S, Bouali-Benazzouz R, Popa RC, Gasser T, Benabid AL, Benazzouz A. Effects of 6-hydroxydopamine-induced severe or partial lesion of the nigrostriatal pathway on the neuronal activity of pallido-subthalamic network in the rat. *Exp.Neurol.* 2007; 205:36–47. [PubMed: 17395181]
7. Casson IR, Pellman EJ, Viano DC. Chronic traumatic encephalopathy in a National Football League player. *Neurosurgery.* 2006; 59:E1152. [PubMed: 17143213]
8. Chen MK, Guilarte TR. Translocator protein 18 kDa (TSPO): molecular sensor of brain injury and repair. *Pharmacol.Ther.* 2008; 118:1–17. [PubMed: 18374421]
9. Cortez SC, McIntosh TK, Noble LJ. Experimental fluid percussion brain injury: vascular disruption and neuronal and glial alterations. *Brain Res.* 1989; 482:271–282. [PubMed: 2706487]
10. Dauer W, Przedborski S. Parkinson's disease: mechanisms and models. *Neuron.* 2003; 39:889–909. [PubMed: 12971891]
11. de Olmos JS, Beltramino CA, de Olmos de LS. Use of an amino-cupric-silver technique for the detection of early and semiacute neuronal degeneration caused by neurotoxicants, hypoxia, and physical trauma. *Neurotoxicol.Teratol.* 1994; 16:545–561. [PubMed: 7532272]
12. Di Monte DA, McCormack A, Petzinger G, Janson AM, Quik M, Langston WJ. Relationship among nigrostriatal denervation, parkinsonism, and dyskinesias in the MPTP primate model. *Mov Disord.* 2000; 15:459–466. [PubMed: 10830409]
13. Dick AD, Broderick C, Forrester JV, Wright GJ. Distribution of OX2 antigen and OX2 receptor within retina. *Invest Ophthalmol.Vis.Sci.* 2001; 42:170–176. [PubMed: 11133863]
14. Duke DC, Moran LB, Pearce RK, Graeber MB. The medial and lateral substantia nigra in Parkinson's disease: mRNA profiles associated with higher brain tissue vulnerability. *Neurogenetics.* 2007; 8:83–94. [PubMed: 17211632]

15. Erlanger DM, Kutner KC, Barth JT, Barnes R. Neuropsychology of sports-related head injury: Dementia Pugilistica to Post Concussion Syndrome. *Clin Neuropsychol.* 1999; 13:193–209. [PubMed: 10949160]
16. Forstl H, Haass C, Hemmer B, Meyer B, Halle M. Boxing-acute complications and late sequelae: from concussion to dementia. *Dtsch Arztebl Int.* 2010; 107:835–839. [PubMed: 21173899]
17. Gash DM, Gerhardt GA, Hoffer BJ. Effects of glial cell line-derived neurotrophic factor on the nigrostriatal dopamine system in rodents and nonhuman primates. *Adv.Pharmacol.* 1998; 42:911–915. [PubMed: 9328046]
18. Grady MS, Charleston JS, Maris D, Witgen BM, Lifshitz J. Neuronal and glial cell number in the hippocampus after experimental traumatic brain injury: analysis by stereological estimation. *J Neurotrauma.* 2003; 20:929–941. [PubMed: 14588110]
19. Greenamyre JT, Hastings TG. Biomedicine. Parkinson's--divergent causes, convergent mechanisms. *Science.* 2004; 304:1120–1122. [PubMed: 15155938]
20. Guilarte TR, Kuhlmann AC, O'Callaghan JP, Miceli RC. Enhanced expression of peripheral benzodiazepine receptors in trimethyltin-exposed rat brain: a biomarker of neurotoxicity. *Neurotoxicology.* 1995; 16:441–450. [PubMed: 8584276]
21. Gundersen HJ. The nucleator. *J.Microsc.* 1988; 151:3–21. [PubMed: 3193456]
22. Gundersen HJ, Bagger P, Bendtsen TF, Evans SM, Korbo L, Marcussen N, Moller A, Nielsen K, Nyengaard JR, Pakkenberg B. The new stereological tools: disector, fractionator, nucleator and point sampled intercepts and their use in pathological research and diagnosis. *APMIS.* 1988; 96:857–881. [PubMed: 3056461]
23. Gundersen HJ, Jensen EB. The efficiency of systematic sampling in stereology and its prediction. *J.Microsc.* 1987; 147:229–263. [PubMed: 3430576]
24. Habgood MD, Bye N, Dziegielewska KM, Ek CJ, Lane MA, Potter A, Morganti-Kossmann C, Saunders NR. Changes in blood-brain barrier permeability to large and small molecules following traumatic brain injury in mice. *Eur.J.Neurosci.* 2007; 25:231–238. [PubMed: 17241284]
25. Hall ED, Andrus PK, Oostveen JA, Althaus JS, VonVoigtlander PF. Neuroprotective effects of the dopamine D2/D3 agonist pramipexole against postischemic or methamphetamine-induced degeneration of nigrostriatal neurons. *Brain Res.* 1996; 742:80–88. [PubMed: 9117424]
26. Hall ED, Bryant YD, Cho W, Sullivan PG. Evolution of post-traumatic neurodegeneration after controlled cortical impact traumatic brain injury in mice and rats as assessed by the de Olmos silver and fluorojade staining methods. *J.Neurotrauma.* 2008; 25:235–247. [PubMed: 18352837]
27. Hall KD, Lifshitz J. Diffuse traumatic brain injury initially attenuates and later expands activation of the rat somatosensory whisker circuit concomitant with neuroplastic responses. *Brain Res.* 2010; 1323:161–173. [PubMed: 20122903]
28. Hebert MA, Gerhardt GA. Normal and drug-induced locomotor behavior in aging: comparison to evoked DA release and tissue content in fischer 344 rats. *Brain Res.* 1998; 797:42–54. [PubMed: 9630504]
29. Hicks R, Soares H, Smith D, McIntosh T. Temporal and spatial characterization of neuronal injury following lateral fluid-percussion brain injury in the rat. *Acta Neuropathol.* 1996; 91:236–246. [PubMed: 8834535]
30. Hoehn MM, Yahr MD. Parkinsonism: onset, progression and mortality. *Neurology.* 1967; 17:427–442. [PubMed: 6067254]
31. Hornykiewicz O. Dopamine (3-hydroxytyramine) and brain function. *Pharmacol.Rev.* 1966; 18:925–964. [PubMed: 5328389]
32. Hornykiewicz O, Kish SJ. Biochemical pathophysiology of Parkinson's disease. *Adv Neurol.* 1987; 45:19–34. [PubMed: 2881444]
33. Hosseini AH, Lifshitz J. Brain injury forces of moderate magnitude elicit the fencing response. *Med.Sci.Sports Exerc.* 2009; 41:1687–1697. [PubMed: 19657303]
34. Hovda DA, Yoshino A, Kawamata T, Katayama Y, Becker DP. Diffuse prolonged depression of cerebral oxidative metabolism following concussive brain injury in the rat: a cytochrome oxidase histochemistry study. *Brain Res.* 1991; 567:1–10. [PubMed: 1667742]

35. Hudson J, Granholm AC, Gerhardt GA, Henry MA, Hoffman A, Biddle P, Leela NS, Mackerlova L, Lile JD, Collins F. Glial cell line-derived neurotrophic factor augments midbrain dopaminergic circuits in vivo. *Brain Res.Bull.* 1995; 36:425–432. [PubMed: 7712205]
36. Huh JW, Widing AG, Raghupathi R. Midline brain injury in the immature rat induces sustained cognitive deficits, bihemispheric axonal injury and neurodegeneration. *Exp.Neurol.* 2008; 213:84–92. [PubMed: 18599043]
37. Hutson CB, Lazo CR, Mortazavi F, Giza CC, Hovda D, Chesselet MF. Traumatic brain injury in adult rats causes progressive nigrostriatal dopaminergic cell loss and enhanced vulnerability to the pesticide paraquat. *J Neurotrauma.* 2011; 28:1783–1801. [PubMed: 21644813]
38. Javitch JA, D'Amato RJ, Strittmatter SM, Snyder SH. Parkinsonism-inducing neurotoxin, N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine: uptake of the metabolite N-methyl-4-phenylpyridine by dopamine neurons explains selective toxicity. *Proc Natl Acad Sci U S A.* 1985; 82:2173–2177. [PubMed: 3872460]
39. Jellinger KA. Recent advances in our understanding of neurodegeneration. *Journal of neural transmission.* 2009; 116:1111–1162. [PubMed: 19707851]
40. Kelley BJ, Lifshitz J, Povlishock JT. Neuroinflammatory responses after experimental diffuse traumatic brain injury. *J Neuropathol Exp Neurol.* 2007; 66:989–1001. [PubMed: 17984681]
41. Kelso ML, Scheff SW, Pauly JR, Loftin CD. Effects of genetic deficiency of cyclooxygenase-1 or cyclooxygenase-2 on functional and histological outcomes following traumatic brain injury in mice. *BMC.Neurosci.* 2009; 10:108. [PubMed: 19719848]
42. Kopin IJ. Monoamine oxidase and catecholamine metabolism. *J.Neural Transm.Suppl.* 1994; 41:57–67. [PubMed: 7931266]
43. Kreutzberg GW. Microglia: a sensor for pathological events in the CNS. *Trends Neurosci.* 1996; 19:312–318. [PubMed: 8843599]
44. Kuhlmann AC, Guilarte TR. The peripheral benzodiazepine receptor is a sensitive indicator of domoic acid neurotoxicity. *Brain Res.* 1997; 751:281–288. [PubMed: 9099816]
45. Langlois, JA.; Rutland-Brown, W.; Thomas, KE. Traumatic brain injury in the United States: emergency department visits, hospitalizations, and deaths. Centers for Disease Control and Prevention, National Center for Injury Prevention and Control; Atlanta, GA: 2004.
46. Lees AJ, Hardy J, Revesz T. Parkinson's disease. *Lancet.* 2009; 373:2055–2066. [PubMed: 19524782]
47. Lifshitz J. Fluid Percussion Injury. In: Chen, J.; Xu, Z.; Xu, XM.; Zhang, J., editors. *Animal Models of Acute Neurological Injuries.* The Humana Press, Inc.; Totowa, NJ: 2008.
48. Lifshitz J, Kelley BJ, Povlishock JT. Perisomatic thalamic axotomy after diffuse traumatic brain injury is associated with atrophy rather than cell death. *J Neuropathol Exp Neurol.* 2007; 66:218–229. [PubMed: 17356383]
49. Lifshitz J, Lisembee AM. Neurodegeneration in the somatosensory cortex after experimental diffuse brain injury. *Brain Struct Funct.* 2011
50. Little KY, McLaughlin DP, Zhang L, Livermore CS, Dalack GW, McFinton PR, DelProposto ZS, Hill E, Cassin BJ, Watson SJ, Cook EH. Cocaine, ethanol, and genotype effects on human midbrain serotonin transporter binding sites and mRNA levels. *Am.J.Psychiatry.* 1998; 155:207–213. [PubMed: 9464199]
51. Litvan I, Halliday G, Hallett M, Goetz CG, Rocca W, Duyckaerts C, Ben-Shlomo Y, Dickson DW, Lang AE, Chesselet MF, Langston WJ, Di Monte DA, Gasser T, Hagg T, Hardy J, Jenner P, Melamed E, Myers RH, Parker D Jr. Price DL. The etiopathogenesis of Parkinson disease and suggestions for future research. Part I. *J Neuropathol.Exp.Neurol.* 2007; 66:251–257. [PubMed: 17413315]
52. Liu M, Choi DY, Hunter RL, Pandya JD, Cass WA, Sullivan PG, Kim HC, Gash DM, Bing G. Trichloroethylene induces dopaminergic neurodegeneration in Fisher 344 rats. *J Neurochem.* 2010; 112:773–783. [PubMed: 19922440]
53. Liu M, Hunter R, Nguyen XV, Kim HC, Bing G. Microsomal epoxide hydrolase deletion enhances tyrosine hydroxylase phosphorylation in mice after MPTP treatment. *J Neurosci Res.* 2008; 86:2792–2801. [PubMed: 18500758]

54. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2⁻(Delta Delta C(T)) Method. *Methods*. 2001; 25:402–408. [PubMed: 11846609]
55. Long-Smith CM, Sullivan AM, Nolan YM. The influence of microglia on the pathogenesis of Parkinson's disease. *Prog.Neurobiol*. 2009; 89:277–287. [PubMed: 19686799]
56. Lotharius J, Dugan LL, O'Malley KL. Distinct mechanisms underlie neurotoxin-mediated cell death in cultured dopaminergic neurons. *J Neurosci*. 1999; 19:1284–1293. [PubMed: 9952406]
57. Martland HS. Punch Drunk. *J Am Med Assoc*. 1928; 91:1103–1107.
58. Maxwell WL, MacKinnon MA, Stewart JE, Graham DI. Stereology of cerebral cortex after traumatic brain injury matched to the Glasgow outcome score. *Brain*. 2010; 133:139–160. [PubMed: 19897544]
59. Maxwell WL, Pennington K, MacKinnon MA, Smith DH, McIntosh TK, Wilson JT, Graham DI. Differential responses in three thalamic nuclei in moderately disabled, severely disabled and vegetative patients after blunt head injury. *Brain*. 2004; 127:2470–2478. [PubMed: 15456707]
60. McGeer PL, Itagaki S, Akiyama H, McGeer EG. Rate of cell death in parkinsonism indicates active neuropathological process. *Ann Neurol*. 1988; 24:574–576. [PubMed: 3239957]
61. McKee AC, Cantu RC, Nowinski CJ, Hedley-Whyte ET, Gavett BE, Budson AE, Santini VE, Lee HS, Kubilus CA, Stern RA. Chronic traumatic encephalopathy in athletes: progressive tauopathy after repetitive head injury. *J Neuropathol Exp Neurol*. 2009; 68:709–735. [PubMed: 19535999]
62. Mendez JS, Finn BW. Use of 6-hydroxydopamine to create lesions in catecholamine neurons in rats. *J Neurosurg*. 1975; 42:166–173. [PubMed: 234524]
63. Minnich JE, Mann SL, Stock M, Stolzenbach KA, Mortell BM, Soderstrom KE, Bohn MC, Kozlowski DA. Glial cell line-derived neurotrophic factor (GDNF) gene delivery protects cortical neurons from dying following a traumatic brain injury. *Restor.Neurol.Neurosci*. 2010; 28:293–309. [PubMed: 20479525]
64. Mokry J. Experimental models and behavioural tests used in the study of Parkinson's disease. *Physiol Res*. 1995; 44:143–150. [PubMed: 8869270]
65. Nakajima H, Uchida K, Yayama T, Kobayashi S, Guerrero AR, Furukawa S, Baba H. Targeted retrograde gene delivery of brain-derived neurotrophic factor suppresses apoptosis of neurons and oligodendroglia after spinal cord injury in rats. *Spine*. 2010; 35:497–504. [PubMed: 20190624]
66. Nedergaard S, Flatman JA, Engberg I. Nifedipine- and omega-conotoxin-sensitive Ca²⁺ conductances in guinea-pig substantia nigra pars compacta neurones. *J.Physiol*. 1993; 466:727–747. [PubMed: 8410714]
67. Neumann J, Gunzer M, Gutzeit HO, Ullrich O, Reymann KG, Dinkel K. Microglia provide neuroprotection after ischemia. *FASEB J*. 2006; 20:714–716. [PubMed: 16473887]
68. Nowak LA, Smith GG, Reyes PF. Dementia in a retired world boxing champion: case report and literature review. *Clin.Neuropathol*. 2009; 28:275–280. [PubMed: 19642506]
69. Omalu BI, DeKosky ST, Hamilton RL, Minster RL, Kamboh MI, Shakir AM, Wecht CH. Chronic traumatic encephalopathy in a national football league player: part II. *Neurosurgery*. 2006; 59:1086–1092. [PubMed: 17143242]
70. Omalu BI, DeKosky ST, Minster RL, Kamboh MI, Hamilton RL, Wecht CH. Chronic traumatic encephalopathy in a National Football League player. *Neurosurgery*. 2005; 57:128–134. [PubMed: 15987548]
71. Omalu BI, Hamilton RL, Kamboh MI, DeKosky ST, Bailes J. Chronic traumatic encephalopathy (CTE) in a National Football League Player: Case report and emerging medicolegal practice questions. *J.Forensic Nurs*. 2010; 6:40–46. [PubMed: 20201914]
72. Pedrosa R, Soares-da-Silva P. Oxidative and non-oxidative mechanisms of neuronal cell death and apoptosis by L-3,4-dihydroxyphenylalanine (L-DOPA) and dopamine. *Br J Pharmacol*. 2002; 137:1305–1313. [PubMed: 12466240]
73. Povlishock JT, Becker DP, Sullivan HG, Miller JD. Vascular permeability alterations to horseradish peroxidase in experimental brain injury. *Brain Res*. 1978; 153:223–239. [PubMed: 687980]
74. Przedborski S. Inflammation and Parkinson's disease pathogenesis. *Mov Disord*. 2010; 25:S55–S57. [PubMed: 20187228]

75. Reeves TM, Phillips LL, Povlishock JT. Myelinated and unmyelinated axons of the corpus callosum differ in vulnerability and functional recovery following traumatic brain injury. *Exp.Neurol.* 2005; 196:126–137. [PubMed: 16109409]
76. Reichert F, Rotshenker S. Deficient activation of microglia during optic nerve degeneration. *J Neuroimmunol.* 1996; 70:153–161. [PubMed: 8898724]
77. Rinder L, Olsson Y. Studies on vascular permeability changes in experimental brain concussion. I. Distribution of circulating fluorescent indicators in brain and cervical cord after sudden mechanical loading of the brain. *Acta Neuropathol.* 1968; 11:183–200. [PubMed: 5709759]
78. Roberts GW, Allsop D, Bruton C. The occult aftermath of boxing. *J.Neurol.Neurosurg.Psychiatry.* 1990; 53:373–378. [PubMed: 2191084]
79. Salvatore MF, Fisher B, Surgener SP, Gerhardt GA, Rouault T. Neurochemical investigations of dopamine neuronal systems in iron-regulatory protein 2 (IRP-2) knockout mice. *Brain Res.Mol.Brain Res.* 2005; 139:341–347. [PubMed: 16051392]
80. Shih MC, Hoexter MQ, Andrade LA, Bressan RA. Parkinson's disease and dopamine transporter neuroimaging: a critical review. *Sao Paulo Med.J.* 2006; 124:168–175. [PubMed: 17119698]
81. Simunovic F, Yi M, Wang Y, Macey L, Brown LT, Krichevsky AM, Andersen SL, Stephens RM, Benes FM, Sonntag KC. Gene expression profiling of substantia nigra dopamine neurons: further insights into Parkinson's disease pathology. *Brain.* 2009; 132:1795–1809. [PubMed: 19052140]
82. Smith L, Tanabe LK, Ando RJ, Kuo CJ, Chung IF, Hsu CN, Lin YS, Klinger R, Friedrich CM, Ganchev K, Torii M, Liu H, Haddow B, Struble CA, Povinelli RJ, Vlachos A, Baumgartner WA Jr. Hunter L, Carpenter B, Tsai RT, Dai HJ, Liu F, Chen Y, Sun C, Katrenko S, Adriaans P, Blaschke C, Torres R, Neves M, Nakov P, Divoli A, Mana-Lopez M, Mata J, Wilbur WJ. Overview of BioCreative II gene mention recognition. *Genome Biol.* 2008; 9(Suppl 2):S2. [PubMed: 18834493]
83. Smith RD, Zhang Z, Kurlan R, McDermott M, Gash DM. Developing a stable bilateral model of parkinsonism in rhesus monkeys. *Neuroscience.* 1993; 52:7–16. [PubMed: 8433810]
84. Stanford JA, Vorontsova E, Surgener SP, Gerhardt GA, Fowler SC. Aged Fischer 344 rats exhibit altered orolingual motor function: relationships with nigrostriatal neurochemical measures. *Neurobiol.Aging.* 2003; 24:259–266. [PubMed: 12498959]
85. Sterio DC. The unbiased estimation of number and sizes of arbitrary particles using the disector. *J.Microsc.* 1984; 134:127–136. [PubMed: 6737468]
86. Streit WJ, Walter SA, Pennell NA. Reactive microgliosis. *Prog.Neurobiol.* 1999; 57:563–581. [PubMed: 10221782]
87. Sulzer D. Multiple hit hypotheses for dopamine neuron loss in Parkinson's disease. *Trends Neurosci.* 2007; 30:244–250. [PubMed: 17418429]
88. Surmeier DJ, Guzman JN, Sanchez-Padilla J, Goldberg JA. The origins of oxidant stress in Parkinson's disease and therapeutic strategies. *Antioxid.Redox.Signal.* 2010a
89. Surmeier DJ, Guzman JN, Sanchez-Padilla J, Goldberg JA. What causes the death of dopaminergic neurons in Parkinson's disease? *Prog.Brain Res.* 2010b; 183:59–77. [PubMed: 20696315]
90. Switzer RC III. Application of silver degeneration stains for neurotoxicity testing. *Toxicol.Pathol.* 2000; 28:70–83. [PubMed: 10668992]
91. Tanno H, Nockels RP, Pitts LH, Noble LJ. Breakdown of the blood-brain barrier after fluid percussive brain injury in the rat. Part I: Distribution and time course of protein extravasation. *J.Neurotrauma.* 1992; 9:21–32. [PubMed: 1619673]
92. Thiffault C, Langston JW, Di Monte DA. Increased striatal dopamine turnover following acute administration of rotenone to mice. *Brain Res.* 2000; 885:283–288. [PubMed: 11102582]
93. Thored P, Heldmann U, Gomes-Leal W, Gisler R, Darsalia V, Taneera J, Nygren JM, Jacobsen SE, Ekdahl CT, Kokaia Z, Lindvall O. Long-term accumulation of microglia with proneurogenic phenotype concomitant with persistent neurogenesis in adult subventricular zone after stroke. *Glia.* 2009; 57:835–849. [PubMed: 19053043]
94. Unterharnscheidt F. A neurologist's reflections on boxing. I: Impact mechanics in boxing and injuries other than central nervous system damage. *Rev.Neurol.* 1995a; 23:661–674. [PubMed: 8597990]

95. Unterharnscheidt F. A neurologist's reflections on boxing. II. Acute and chronic clinical findings secondary to central nervous system damage. *Rev.Neurol.* 1995b; 23:833–846. [PubMed: 7497249]
96. Unterharnscheidt F. A neurologist's reflections on boxing. III. Vascular injuries. *Rev.Neurol.* 1995c; 23:847–855. [PubMed: 7497250]
97. Unterharnscheidt F. A neurologist's reflections on boxing. IV. Late and permanent brain damage. *Rev.Neurol.* 1995d; 23:1013–1026. [PubMed: 8556584]
98. Unterharnscheidt F. A neurologist's reflections on boxing. V. Conclude remarks. *Rev.Neurol.* 1995e; 23:1027–1032. [PubMed: 8556585]
99. Wang CX, Shuaib A. Involvement of inflammatory cytokines in central nervous system injury. *Prog.Neurobiol.* 2002; 67:161–172. [PubMed: 12126659]
100. West MJ, Ostergaard K, Andreassen OA, Finsen B. Estimation of the number of somatostatin neurons in the striatum: an in situ hybridization study using the optical fractionator method. *J.Comp Neurol.* 1996; 370:11–22. [PubMed: 8797153]
101. Witgen BM, Lifshitz J, Smith ML, Schwarzbach E, Liang SL, Grady MS, Cohen AS. Regional hippocampal alteration associated with cognitive deficit following experimental brain injury: a systems, network and cellular evaluation. *Neuroscience.* 2005; 133:1–15. [PubMed: 15893627]
102. Yan HQ, Ma X, Chen X, Li Y, Shao L, Dixon CE. Delayed increase of tyrosine hydroxylase expression in rat nigrostriatal system after traumatic brain injury. *Brain Res.* 2007; 1134:171–179. [PubMed: 17196177]
103. Yoshino A, Hovda DA, Kawamata T, Katayama Y, Becker DP. Dynamic changes in local cerebral glucose utilization following cerebral conclusion in rats: evidence of a hyper- and subsequent hypometabolic state. *Brain Res.* 1991; 561:106–119. [PubMed: 1797338]
104. Zigmond MJ. Do compensatory processes underlie the preclinical phase of neurodegenerative disease? Insights from an animal model of parkinsonism. *Neurobiol.Dis.* 1997; 4:247–253. [PubMed: 9361301]

Highlights

- Substantia nigra (SN) shows neurovascular vulnerability to single moderate diffuse brain injury
- By one month, significant reduction in SN neurons, a majority of which are TH-positive, without reduction in dopamine metabolism or synthesis
- Injury-induced vascular compromise leads to sustained microglial activation in SN

Figure [1]. GIEMSA

Neuronal cell loss, without atrophy, is evident in the SN after diffuse traumatic brain injury induced by midline FPI. Giemsa stained coronal sections from sham (A) and 28 day (B) mFPI adult male rats. Stereological estimates of neuronal number using the optical fractionator technique in the SN reveal a significant 44% neuronal loss by 28 days after moderate midline FPI compared to uninjured sham (C). Normalizing to regional volume, there is a significant decrease in neuronal density at 28 days compared to sham and 7 day rats (D). As estimated using the nucleator, neuronal nuclear volumes of the remaining neurons are similar across groups (E). *, $p < 0.05$ compared to sham; †, $p < 0.05$ compared to 7d FPI. Scale bar = 250 μm . Mean $\pm$ SEM.

Figure [2]. DA/DOPAC/HVA

Whole tissue measurements of DA and DA metabolites by HPLC-EC. At 28 days after FPI, whole tissue levels of DA show no significant injury-induced differences compared to uninjured sham (A). Moderate FPI results in a non-significant reduction in DOPAC (B) and a significant reduction of the dopamine metabolite HVA (C) compared to uninjured sham. The rate of dopamine turnover (DOPAC + HVA / DA) reveals a statistically significant decrease at 28 days post-injury compared to sham (D). *, $p < 0.05$ compared to sham. Mean $\pm$ SEM.

Figure [3]. TH

Tyrosine hydroxylase (TH) content in the SN in sham (A) and 28 days (B) fluid percussion injured (FPI) animals. There was no change in the percent of the SN positively stained with TH from sham and 28 day FPI rats (C). No significant differences were found in relative TH gene expression in uninjured and FPI SN (D). Stereological estimates showed a significant 25% reduction of TH-positive neurons in the SN. Scale bar = 300 μm . *, $p < 0.05$ compared to sham. Mean $\pm$ SEM.

Figure [4]. Silver-Stained Sections

Time dependent neurodegeneration indicated by silver stained sections of SN from sham (A), 7 day (B) and 28 day (C) FPI rats. Quantification of silver stain demonstrates a significant increase in neurodegeneration at 7 days post-injury compared to sham, which continue to increase significantly from 7 days to 28 days post-injury (D). *, $p < 0.05$ compared to sham; †, $p < 0.05$ compared to 7d FPI. Scale bar = 300 μm. Mean ± SEM.

Figure [5]. RTI-55

$[^{125}\text{I}]\text{-RTI-55}$ radioligand binding in uninjured sham (A), 7 day (B) and 28 day (C) FPI rat brains to indicate DAT density. Higher intensity $[^{125}\text{I}]\text{-RTI-55}$ binding is localized to the SN. Quantification of $[^{125}\text{I}]\text{-RTI-55}$ radioligand binding density indicates no significant change in DAT density (D). Relative gene expression of DAT indicates no significant change in DAT mRNA concentrations (E). Scale bar = 5 mm. Mean $\pm$ SEM.

Figure [6]. IgG

Vascular permeability identified by IgG immunostaining in the SN after sham (A) and FPI (B, C). Insets indicate the multi-focal occurrence of vascular compromise 1 and 2 days following diffuse brain injury (scale bar = 500 μ m). High power photomicrographs indicate the vascular compromise as star-burst like immunostaining in the vicinity of cerebral vessels (scale bar = 100 μ m). No IgG staining was observed at the 7 day or 28 day time points (not shown).

Figure [7]. Iba-1

Microglia in the SN are labeled by immunohistochemistry for Iba-1. The highly ramified microglia with spherical cell bodies in sham rats (A) become less ramified with swollen cell bodies at 2 days post-injury (B). Over the 7 day (C) and 28 day (D) post-injury time course, microglial morphology resumes the higher ramified phenotype, but to a lesser extent than in sham rats. Scale bar = 20 μ m.

Figure [8]. PK-11195

$[^3\text{H}]$ -PK-11195 radioligand binding to 18kDa TSPO (translocator protein) images from sham (A), 2 day (B), 7 day (C) and 28 day (D) FPI rats. Higher intensity $[^3\text{H}]$ -PK-11195 binding indicates increased TSPO receptor density, indicative of microglial activation in the SN. Binding in the cerebral cortices, dentate gyrus and lateral thalamus are also seen (not quantified). Quantification of $[^3\text{H}]$ -PK-11195 radioligand binding density indicates significant increases TSPO binding density at 7 days post-injury (E). Relative TSPO and CD45 gene expression indicate increased mRNA levels by 7 days post-injury, which become significant at 28 days post-injury compared to uninjured sham and 7 days post-injury (F). *, $p < 0.05$ compared to sham; †, $p < 0.05$ compared to 7d FPI. Scale bar = 5 mm. Mean $\pm$ SEM.