

AN ANIMAL MODEL OF SPORT RELATED CONCUSSIVE BRAIN INJURY

Hayde Bolouri

Department of Medical Chemistry and Cell Biology
Institute of Biomedicine, Sahlgrenska Academy
Sweden



UNIVERSITY OF GOTHENBURG

2008

Printed in Sweden

by *INTELLECTA*, Gothenburg 2008

ISBN 978-91- 628-7589-3

Cover picture shows the impact setup

CONTENTS

ABSTRACT	4
LIST OF PAPERS.....	6
POPULÄRVETENSKAPLIG SAMMANFATTNING PÅ SVENSKA.....	7
INTRODUCTION	8
<i>Background</i>	8
<i>Animal model for brain injury</i>	9
<i>Effects of concussion on brain structure</i>	9
<i>Effects of concussion on brain function</i>	10
AIMS	11
MATERIALS AND METHODS	12
<i>Experimental animals</i>	12
<i>Impact procedure</i>	12
<i>Perfusion and brain dissection</i>	13
<i>Immunohistochemistry</i>	13
<i>Assessment of behavior</i>	14
A. Morris Water Maze (MWM).....	14
B. Open field activity	15
<i>Statistical analyses</i>	15
RESULTS	16
<i>Concussion model (I)</i>	16
<i>Neuronal cytoskeleton (II)</i>	17
<i>Glial cell reactivity (II)</i>	17
<i>Cognitive dysfunction (III)</i>	18
DISCUSSION	19
<i>Immunohistochemistry</i>	19
<i>Morris Water Maze</i>	20
<i>Comparison with other TBI models</i>	20
<i>Immunohistochemistry of neurofilaments</i>	21
<i>NF-200 and DAI</i>	21
<i>Immunohistochemistry of GFAP</i>	22
<i>The Morris Water Maze test and the hippocampus</i>	23
<i>Exploratory activity</i>	23
<i>Limitations</i>	24
<i>Significance and outlook</i>	24
ACKNOWLEDGMENTS.....	25
REFERENCES	26

An animal model of sport related concussive brain injury

Hayde Bolouri

Institute of Biomedicine, University of Gothenburg, Sweden

ABSTRACT

A new animal model for concussion of the type seen in professional football was developed, since current animal models did not simulate these conditions. The model is characterized by a high velocity-low mass impact to the head of a freely moving object. Structural damages and functional effects of the model have been investigated.

Paper I describes the rat model. A pneumatically driven projectile impacted the temporal region of the head. A 50 g projectile matches the concussions in football players scaled to the rat. Exposures were also performed with a 100 g impactor. The pressure accelerated the projectile to velocities of 7.4 m/s, 9.3 m/s and 11.2 m/s. The head was protected with a padded aluminum helmet. A small accelerometer was attached on the opposite side of the head, inline with the impact, for recording the acceleration of the head. Rats were exposed to a single or repeated (3, with 6 hour intervals) impacts and were sacrificed 1, 4 or 10 days later. Peak head acceleration, ΔV , duration and energy transfer were determined. Brains were perfused and surface injuries identified. Skull fractures were never found. Impact velocity and head ΔV and acceleration were within 1% and 3% of the target.

In **paper II**, neuronal injury was assessed with immunohistochemistry for NF-200, the heaviest neurofilament subunit, and GFAP, an intermediate filament protein in astrocytes. Hemorrhages were visualized with unspecific peroxidase. NF-200 immunoreactivity was accumulated in neuronal perikarya and was reduced in the axons 10 days after impact. Reactive astrocytes were found in the midline regions of the cerebral cortex and periventricularly. Erythrocyte-loaded blood capillaries indicated brain edema in regions of the cerebral cortex, brain stem and cerebellum. A single impact at 7.4 and 9.3 m/s with the 50 g projectile resulted in minimal neuronal injury and astrocytosis. Repeated impacts with the 100 g projectile at 11.2 m/s and 9.3 m/s led to injury bilaterally in the cerebral cortex, subcortical white matter, hippocampus CA1, corpus callosum and the striatum. The pattern of injury is suggestive of Diffuse Axonal Injury (DAI).

In **paper III**, cognitive function and exploratory behavior were investigated following repeated head impacts. Rats were trained daily for 6 days in the Morris Water Maze. The time of latency to find a hidden platform was reduced from 50 secs on day 1 to 15 secs on day 6. They were then exposed with the 50 g or the 100 g projectile at 9.3 or 11.2 m/s. Spontaneous exploratory activity was assessed with the open field test 2-4 days and 1 and 2 weeks after impacts with the 50 g projectile at 9.3 and 11.2 m/s. The results showed that rats exposed at 11.2 m/s (x3) with the 50 g projectile or 9.3 m/s (x3) and 11.2 m/s (x2) with the 100 g projectile had a significantly increased time of latency to the platform, while those exposed with the 50 g at 9.3 m/s did not differ from the controls. Rats impacted with 50 g (x3) at 9.3 or 11.2 m/s showed a significant decrease in spontaneous exploratory activity.

In conclusion, the model fulfilled the conditions of concussion in the freely moving animal, without preparatory surgery, still with good reproducibility. Some aspects of the neuropathology and functional effects were investigated and both showed dose-response effects. The functional changes were cognitive deficits and reduced exploratory activity.

Key words: Animal model, football, concussion, brain injury, neuropathology, cognitive function.

LIST OF PAPERS

This thesis is based on the following papers:

- I. David C. Viano, Anders Hamberger, Hayde Bolouri, Annette Säljö.
Concussion in Professional Football: Animal Model of Brain Injury – Part 15.
Submitted for publication in Neurosurgery
- II. Anders Hamberger, David C. Viano, Annette Säljö, Hayde Bolouri.
Concussion in the NFL: Morphology of brain injuries in the NFL concussion model – Part 16
Accepted for publication in Neurosurgery
- III. Hayde Bolouri, Anders Hamberger, David C. Viano and Annette Säljö.
Concussions in the NFL: Cognitive impairment following repeat head impacts – Part 18
Submitted for publication in Neurosurgery

POPULÄRVETENSKAPLIG SAMMANFATTNING PÅ SVENSKA

I samband med sport och rekreation inträffar enbart i USA över 300 000 hjärnskador per år. Av dessa är 95 % lätta skador (hjärnskakning). De flesta sportrelaterade skadorna inträffar inom amerikansk fotboll. Många fotbollsspelare i de professionella ligorna i USA lider bl.a. av huvudvärk, minnesrubbingar och yrselattacker. Besvären är oftast övergående och efter en veckas vila kan i allmänhet spelarna återgå till verksamheten. Oron över effekterna av upprepade hjärnskakningar har lett till att National Football League (NFL) sedan mitten av 1990-talet stimulerat till ett antal forskningsprojekt. Genom analyser av videoinspelningar från fotbollsmatcher och rekonstruktioner med testdockor har man fått fram en karakterisering av krafterna vid kollisioner mellan spelare. NFL har även initierat utveckling av djurmodeller för fotbollsrelaterad hjärnskakning för mer kontrollerade studier av skademekanismer, behandling och tillfrisknande. Avhandlingen beskriver en modell för skallskador, i vilken de slag mot huvudet, som spelarna utsätts för i professionell fotboll i USA och som ofta medför hjärnskakning simuleras på råttor. Modellen har sannolikt relevans även för andra kontaktsporter.

Delarbete 1 beskriver modellen. Huvudets önskade acceleration och maximala hastighet uppnåddes med en projektil med massan 50 g, som träffade råttans hjälmkyddade huvud i tinningregionen med en hastighet av 9,3 meter per sekund. Även effekten av hastigheterna 7,4 och 11,2 meter per sekund och tre upprepade (var sjätte timme) slag undersöktes. Huvudets acceleration och hastighet mättes rutinmässigt med en liten accelerometer, som är fäst på huvudet. Dessutom registreras projektilhastigheten och huvudets hastighet med ett fotocellssystem samt filmas med höghastighetsvideo. Slaget ledde aldrig till skada på skallbenet. En mycket liten blödning kunde ibland observeras över den hjärnregion, som träffas av slaget. (Insänt till Neurosurgery).

Delarbete 2 är en karakterisering av den mikroskopiska skadebilden i hjärnan vid olika tidpunkter efter slaget. Hjärnan undersöks avseende förekomst av blödningar genom färgning av röda blodkroppar. Avgränsade 0,1–1 mm stora blödningar ses i slagregionen. Hjärnans celler studeras med immunteknik, som hos nervceller visualiserar tunna trådar i cellskelettet (neurofilament) samt liknande strukturer i hjärnans stödjeceller (gliafilament). Tio dagar efter slaget ser man en redistribuering av vissa komponenter i neurofilamenten i hjärnbarkens och vissa andra regioners nervceller. Stödjecellerna aktiveras, dvs ökar i storlek får, efter en dryg vecka, en ökad ansamling av gliafilament, dels i slagregionen samt i flera andra områden i hjärnan. Stödjecellernas skademönster är mycket likt det man ser hos människa efter slag mot huvudet (Accepterat av Neurosurgery).

Delarbete 3 är en analys av hur skadan påverkar hjärnans minnes- och inlärningsfunktioner. De utvärderades med Morris Water Maze metoden, i vilka djuren simmande lär sig att finna en liten plattform, dold några mm under en vattenyta i en rund ”swimming-pool”. Efter 5-6 dagars träning finner råttan plattformen inom 15 sekunder medan det tog 60 sekunder första dagen. Efter träningsperioden utsätts djuren för slag mot huvudet och en dag senare testas åter deras förmåga att finna plattformen. Exponering för 3 slag med 50 och 100 g projektiler och en hastighet av 9,3 och 11,2 m/sek gav en ökning av tiden, som åtgår för att finna plattformen. (Insänt till Neurosurgery).

INTRODUCTION

Background

Traumatic brain injury (TBI) occurs when an external physical force impacts the head, either causing the brain to move within the intact skull or damages the brain by fracturing the skull (McCrory et al., 2005). Various types and levels of impact damage the brain differently. TBI may acutely alter the state of consciousness and, with time, impair cognitive abilities, behavior and/or physical function. Over one million new cases of TBI occur every year in the USA. Of these, an estimated 300 000 cases take place in the setting of sports and recreation, 95% being mild traumatic brain injury (MTBI) or concussion, (Kelly 1997). MTBI leads to a short deterioration in neural function that may or may not involve loss of consciousness (Kelly et al., 1997). It may result from neuropathological changes, but the acute clinical symptoms reflect a functional disorder rather than a structural damage. MTBI is generally associated with normal structural neuroimaging (Aubry et al., 2002; McCrory et al., 2005).

Football accounts for the highest proportion of MTBI (Powell et al., 1999). Over 60% of the players in professional football (National Football League, NFL) have clinical signs and symptoms of MTBI (Pellman et al., 2004; Pellman et al., 2004; Pellman et al., 2005). Headache is most common and found in 60% of the NFL players (Sallis et al., 2000). Other symptoms are memory problems (40%), dizziness (40%), cognitive dysfunction (25%), tremor, irritability, anxiety, depression, tinnitus, other auditory symptoms, neck and back pain, anosmia and visual problems (Collins et al., 1999; McCrea et al., 2002; Ponsford et al., 2008). Concussed football players are mostly free of symptoms after one week and return to the game, although it is not known whether this is sufficient time for recovery (Macciocchi et al., 1996; Collins et al., 1999; Makdissi et al., 2001; Bleiberg et al., 2004). TBI may also lead to lasting neurological effects that are not immediately evident (Mortimer et al., 1991). There is an increased risk of depression (Holsinger et al., 2002), Alzheimer's disease and other dementias (Plassman et al., 2000) many years after TBI. Less is known about the consequences of MTBI in this respect.

NFL formed a committee on MTBI in 1994 in response to concerns regarding the effects of concussions on the players. A series of projects were started on concussion biomechanics, based on analysis of game videos and laboratory reconstructions with test dummies. The results include impact velocities, head velocity ΔV , acceleration and location and direction of impacts (Pellman et al., 2003; Pellman et al., 2003). The

committee also saw the need for an animal model, which would allow more controlled studies of injury mechanisms, therapy and recovery. A project on the development of an animal model for football concussion was given to our group in the year 2000. The model should simulate the translational, high speed head impact to a freely moving subject. The model should not require preparatory surgery and not cause musculo-skeletal injury. Techniques for scaling impact conditions in the game videos from man to animal should be employed (Ommaya et al., 1967; Gutierrez et al., 2001).

Animal model for brain injury

Available animal models of TBI do not fulfill the requirement of high impact velocity and rapid change in head ΔV without injuring the skull bone. Furthermore, they have mostly limited control of the biomechanical parameters in each experiment. Lateral fluid percussion involves surgical preparation and the impact is achieved by injection of fluid into the epidural space (Sullivan et al., 1976). Moreover, the highest speed of impact used in lateral fluid percussion is lower than that required for concussion in football. In the weight drop techniques, the head is not freely moving but squeezed between the impacting mass and the supporting table (Foda et al., 1994; Marmarou et al., 1994). Impacting at the higher levels employed in Marmarou's technique lead to acute neurological deficits, weight loss, hypotension, diffuse edema and motor deficits (Adelson et al., 1996). Techniques which involve air-driven pistons have the versatility to simulate concussion, since they offer a wide range of impact velocity. The technique of Nilsson et al (Nilsson et al., 1977) combines an air driven piston with the freely moving intact head/animal, which is impacted on the top of the head. The impact in football is mostly in the frontal or temporal region. Other techniques involving air-driven pistons require surgical preparation (Lighthall 1988; Adelson et al., 1996).

This thesis presents a new TBI model, which meets the conditions of concussion in football scaled from man to rat and provides high reproducibility and reliability. The thesis also presents an initial characterization of the effect of the impact, first on structural components in the brain and secondly on the neural function associated with cognitive ability.

Effects of concussion on brain structure

Macroscopically, the brain is composed of a large number of regions, each with its specific function. Microscopically, the brain contains approximately 50 billion nerve

cells or neurons, which are specialized to receive, conduct and transmit impulses. Neurons have certain morphological features in common, which reflect that nervous tissue functions as a communication system (Heimer 1995). The cell body generally comprises a minor portion of the neuron, while its axonal process often dominates the cell volume. There is a continuously ongoing transport of proteins and waste products into the axon (Droz et al., 1962) from the cell body, which contains the normal set-up of cell organelles. The intermediate filaments (neurofilaments, NF), which are components of the cytoskeleton, contain different subunits with a molecular weight in the range 61-115 kDa. The brain also contains cells with supportive function, the glial cells, i.e. astrocytes, oligodendrocytes and microglial cells. The most abundant supporting cell is the astrocyte, which is physically supportive to neurons and performs essential tasks for their function. Its intermediate filaments have a major structural component, the Glial Fibrillary Acidic Protein (GFAP).

Macroscopically, a TBI induced brain injury may be seen as hemorrhages in the meninges and/or in the brain tissue. It may also involve destruction of brain tissue. Evaluation of injury in various brain regions at the microscopical level is done with histological, immunohistochemical, autoradiographic or similar techniques. Neuronal death may be visualized with various staining techniques (Gavrieli et al., 1992, Hall, 2008 #695; Hall et al., 2008). TBI may involve injury to specific cell organelles, such as mitochondria (Lifshitz et al., 2004) and neurofilaments (Droz et al., 1962). Neurodegeneration (Hall et al., 2008) and diffuse axonal injury (DAI), a frequent injury in TBI, have distinctive pathological features Gennarelli (Gennarelli et al., 1982; Adams et al., 1992).

Astrocytes are activated in response to neuronal injury or death, inflammation or the presence of plasma components in the brain tissue (Stoll et al., 1998; Raivich et al., 1999). Reactive gliosis is characterized by hyperplasia and hypertrophy of the astrocytes. Activated astrocytes are also characterized by an increase of the immunodetectable glial fibrillary acidic protein (GFAP; (Yu et al., 1993). Activated astrocytes migrate towards the injury and surround it with a scar, which can be detrimental for neurite growth (Reire 1986; Davies et al., 1997; Fawcett et al., 1999).

Effects of concussion on brain function

There are at least as many methods for the study of brain function as for structural studies. They test the function of specific sensory and/or motor systems, each having its site in the brain. We decided to monitor cognitive function in the model, since there is a high frequency of memory problems and cognitive dysfunction in professional

football players. The brain region, which is essential for cognitive function is the hippocampal formation (Eichenbaum 1997). Cognitive dysfunction and hippocampal damage (Smith et al., 2006) with loss of pyramidal neurons have been reported after TBI (Hamm et al., 1993; Goodman et al., 1994). The Morris water maze (Morris 1984) is one of the most frequently used tools in behavioral neuroscience, explicitly memory and learning in the laboratory rat. In addition to the cognitive function, we analyzed the spontaneous exploratory activity in an open field (Larsson et al., 2002). Rodents usually explore their environment and a brain damage may disturb this behavior (Mikulecka et al., 2004).

AIMS

The aim of this thesis was to increase the understanding of concussion biomechanics in professional football. Its specific aims were:

- I. To develop an animal model simulating concussion biomechanics in professional football players.
- II. To characterize the brain after the concussion, macroscopically and at the cellular level.
- III. To characterize the performance of the animal with respect to cognitive function after the concussion.

MATERIALS AND METHODS

Experimental animals

Adult male Wistar rats (Charles River, Sulzfeld - Germany) with a body weight of 250-370 g (I and II) and 230-330 g (III) were used. They were 7-11 weeks old, according to the Charles River Laboratory (www.criver.com, 2008). The animals were housed in cages (Macrolon IV, 24 x 42 x 15 cm), maximally 4 per cage in a colony room for one week prior to the experiments. They had free access to water and pelleted food (R 34; Lactamin, Stockholm, Sweden). Room temperature ($20 \pm 1^\circ \text{C}$) and humidity (55%) were kept constant. The animal quarters had a light and dark cycle changing at 6 a.m. and 6 p.m. All experiments were performed during the light phase. The study was approved by the Gothenburg Animal Experiments Ethical Committee (A 282/02, 163/04) and performed according to the guidelines for laboratory animal care.

Impact procedure

The velocity of the projectile was proportional to the pressure set in the accumulator. The pressure had to be increased approximately twice to accelerate a 100 g projectile to a velocity similar to that of a 50 g projectile. The impact on the rat with a 50 g projectile was in accordance with that of the striking-to-struck player in football (Viano et al., 2005). Exposure to head impact was carried out under a general anesthesia. The animals were allowed to breathe spontaneously. The heart rate was recorded. Both sides of the head were shaved. The head was equipped with a small accelerometer on the right side and a padded aluminum plate (the "helmet") on the left side. The head was impacted in the left temporal region with a free-moving projectile, which was accelerated pneumatically. The animals received either one or 3 exposures (6 h interval) at impact velocities of 7.4, 9.3 or 11.2 m/s. Sham animals received only anesthesia. In the main series, the rats were impacted with a 50 g projectile. In addition, a series was carried out with a 100 g projectile. The animals were sacrificed after 1, 4 or 10 days.

Perfusion and brain dissection

Twenty seven rats exposed with the 50 g projectile, 31 rats with the 100 g projectile and a total of 14 sham exposed animals were used for histology/immunohistochemistry. They were sacrificed by transcardial perfusion at room temperature, first with 200 ml phosphate buffered saline (PBS; pH 7.4), and then with 200 ml phosphate buffered formalin, prepared by alkaline hydrolysis of paraformaldehyde (3.8 % w/v; pH 7.2-7.4). The brains were photographed in situ and/or after being removed from the skull. The removed brains were post-fixed in formalin for 2 h at 4° C. They were then stored at 4° C in PBS, containing 20 % (w/v) sucrose and 0.1 % (w/v) sodium azide for at least one week. A block of the brain was cut from bregma 6 mm caudally. It was moved to a 20 % (w/v) sucrose solution mixed with an equal volume Tissue-Tek® (O.C.T. compound Tissue – Tek®, Sakura Finetek Europe B.V. Hoge Rijndijk 48 a, 2382 AT Zoeterwoude, The Netherlands). The slice was then frozen by being held for 6 min approximately 10 mm over the surface of liquid nitrogen in a thermos. Coronal sections (25 µm) were cut from the frozen brain block in the Paxinos plate 21 (Paxinos et al., 1982) region with a cryostat microtome (Leica Microsystems, CM 3050S, Heidelberg, Germany). The sections were then stored in a tissue cryoprotectant solution (25% ethylene glycol, 25% glycerol and 0.1 M phosphate buffer, Naylor 2005) at –20°C until used for immunohistochemistry.

Immunohistochemistry

The sections were used free-floating and rinsed in fresh PBS to remove the cryoprotectant. They were then treated in PBS containing 0.6 % H₂O₂ to block endogenous peroxidase activity and incubated in NF-200 (NCL-NF-200-N52; 1:100, NOVOCASTRA Laboratories Ltd, UK) or GFAP (NCL-GFAP-GA5; 1:2000, NOVOCASTRA Laboratories Ltd, UK) for immunoreactivity. For visualization of unspecific peroxidase activity, the section were rinsed in PBS and incubated with DAB (SK-4100 substrate kit, Vector Laboratories, Inc. Burlingame, CA, USA). All sections were coded and evaluated independently by three investigators. We also tested antisera against the NF-68 epitope of neurofilaments (NCL-NF68-DA2, NOVOCASTRA Laboratories Ltd, UK), the beta amyloid precursor protein (Lewen et al., 1995) and ribonucleotide reductase (Zhu et al., 2003). All sections were coded and evaluated independently by 3 investigators.

Assessment of behavior

A. Morris water maze (MWM)

Four groups of rats (16 animals in each) were used to study spatial memory and learning in a behavioral test, the MWM (Morris et al., 1982). The tests were performed in a circular pool made of black plastic material. The pool had a diameter of 1.6 m, a height of 0.6 meter and was centered in a room measuring 2.5 x 2.5 m. A circular Plexiglas platform with a diameter of 100 mm was mounted on a stand to keep it approximately 200 mm above the floor in one of the pool quadrants. The water level was 10 mm above the platform. The water in the pool ($22\pm 1^{\circ}\text{C}$) was clear and contained no additions. An overhead camera, which was connected to a computer, tracked the animal's progress and calculated the time spent in each area of the pool (software, HVS Image, 2020 Plus Tracking system, HVS Image Ltd, Buckingham, UK). The walls and ceiling of the room were covered with dark textiles in order to separate the pool area from the computer station and to leave the animal undisturbed during the tests. There were a few maze cues and two lamps on each of two opposite walls.

The animals were handled daily for 6 days prior to the experiments at the same time of the day in order to get used to the facilities and the person in charge. On the first training day, the rats were given an acclimatization session in the water for 120 secs. The platform was then visible 5 mm above the water surface. An acquisition period with a hidden platform started on the next day. The rat was gently placed in the water at the periphery of the pool, facing its wall, from one of four positions (North, East, South, and West). Each training session consisted of 4 trials, one from each position in random order. In case the animal had found the platform, it was allowed to rest on top of it for 15 secs. Then it was placed in a cage, covered by dark cloth. It was left in the cage for an intertrial interval of approximately 3 min and was then returned to the water for the next trial. During testing, the person in charge remained behind the curtain, in order to avoid being a landmark cue. If a rat failed to locate the platform within 60 secs, it was guided on to it and allowed to rest on the platform for 15 secs. Identical training sessions were performed once daily, at the same time of the day for 6 consecutive days. The latency to platform had then decreased to approximately 20 % of the initial time. After each session, the rat was dried under a fan heater before being returned to the home cage.

After completion of the training period, the animals were randomly divided into groups to be exposed to (3 repeated impacts) or controls. Exposition took place on the

7th day. The 8th day was a recovery day (Fujimoto et al., 2004). On the 9th day, the rats underwent a test session, identical to the training sessions. The training and testing sessions always started at 8 am. This time is within the animal's activity period (darkness) and was chosen to avoid effects on sleeping patterns. Testing in the MWM during an animal's inactive phase affects performance (Valentinuzzi et al., 2004).

B. Open field activity

Stress/anxiety influences the spontaneous exploratory activity of the rat (Larsson et al., 2002), which we assessed with the open field test (Giulian et al., 1975), a test frequently used in behavioral neuroscience (O'Connor et al., 2003). The recorded time of activity (5 to 10 min) depends on the test paradigm (Vallee et al., 1997; O'Connor et al., 2003). We decided to record the activity during a 10 min period, but analyzed separately the first and second 5 min periods. Parameters, such as path length, total entries, movement patterns and time spent in each square were calculated (software, HVS Image, 2020 Plus Tracking system, HVS Image Ltd, Buckingham, UK). Here, the total entries means the number of square the animal entered, with each square being eligible to be counted more than once should the animal leave the square and then return. The entry of the rat into the squares of the periphery was also recorded. Path length is the total distance traveled by the rat.

Briefly, a one-meter square box with 450 mm high black paneled walls was placed under a video camera which was connected to a computer. The base of the box was subdivided by the software into 25 equal squares, each being 200 mm x 200 mm. A baseline of activity was determined before the impact to the head. Two groups of rats were used to assess the spontaneous exploratory activity 2 days after receiving repeated (3) impacts at 9.3 or 11.2 m/s.

Statistical analyses

Chi-square and student t-test (I), independent paired or unpaired t-test and two-way ANOVA (III) were used. The results in III are expressed as means \pm S.E.M. Effects are considered significant at p -values < 0.05 .

RESULTS

Concussion model (I)

The aim of the first report (I) was to develop a rat model that fulfilled, as well as possible, the criteria for concussion in professional football. Impact velocity, head ΔV , acceleration and pulse duration are determinable and controlled. The first goal was to find the optimal weight of the projectile. A range of weights (10-300 g) was tested. A 50 g projectile gave a head ΔV , acceleration and duration, which closely matched the results from video reconstructions and man to rat scaling.

The combination of a freely moving animal, reproducibility of the position of the animal and the point of impact was solved by keeping the “helmet” protected head in a place with the help of magnets. The magnets ensured reproducible position and did not interfere with the characteristics of the impact. The input from the accelerometer and the speed traps were saved from every impact.

There were no skull fractures or tissue tears on the brain surface, in agreement with the situation after concussion in football. Up to 1.0 mm large petechial bleedings were seen on the brain surface under the point of impact. They increased in size and frequency with increasing impact velocity, repeated impact and higher mass of the projectile. After repeated exposures of the animals, petechial bleedings could be seen at some distance from the impact site, i.e. more inferiorly on the temporal lobe. No hemorrhages were seen contralateral to the impact site or elsewhere in the cerebrum, cerebellum or brain stem.

Microscopical examination showed that the petechial bleedings on the brain surface were hemorrhages in the superficial brain parenchyma. Erythrocyte-filled blood vessels could be seen in the regions around the hemorrhages. After impact with a 100 g projectile, erythrocyte-filled blood vessels could also be seen in other regions of the cerebral cortex and the cerebellum. The meningeal regions over the petechial hemorrhages appeared to be intact. Intraventricular hemorrhages were never observed.

Neuronal cytoskeleton (II)

Coronal sections from the somatosensory cortex, frontoparietal cortex, midline (cingulum), hippocampus CA 1 and the dentate gyrus were routinely monitored. In the cerebral cortex of control animals, axons and dendrites of neurons throughout layers II-VI were selectively immunoreactive for NF-200, while little immunoreactivity was seen in the neuronal perikarya. In the hippocampus of control animals, the perikarya of the CA 1-3 pyramidal neurons and the granule cells of the dentate gyrus lacked NF-200 immunoreactivity, whereas axons were distinctly outlined in the superior and inferior regions.

In animals exposed 10 days, but not one or 4 days earlier, neuronal perikarya in the cerebral cortex were generally intensely stained. Perikarya of CA1 pyramidal neurons also developed increased immunoreactivity. A small increase in NF-200 immunoreactivity was seen in neuronal perikarya in the cortical layers III-V on the contralateral as well as the ipsilateral side in animals exposed repeatedly at 7.4 or 9.3 m/s.

Glial cell reactivity (II)

In the brains of control animals, GFAP-positive astrocytes were unevenly distributed. In the cerebral cortex, they were confined to the outer layers, while the middle and deep layers had weakly stained or unstained astrocytes. The brains of animals exposed to higher impact velocity had an increased frequency of GFAP positive astrocytes. Here, the astrocytes also had larger perikarya and thicker cell processes (reactive astrocytes). Reactive astrocytes were detected in the subcortical white matter of the motor cortex, the midline of the corpus callosum, hippocampus CA1 and periventricularly in the striatum. The ipsilateral and contralateral sides did not differ markedly with respect to the frequency of reactive astrocytes. The 100 g projectile increased the frequency of reactive astrocytes.

Other markers

We were not able to obtain consistent results with any of the antisera against the NF-68 epitope of neurofilaments, the beta amyloid precursor protein or ribonucleotide reductase.

Cognitive dysfunction (III)

The performance of the group exposed with the 50 g projectile at 9.3 m/s (x3) did not differ from that of the controls, however a significant increase in the performance was seen in animals exposed at 11.2 m/s (x3) with 50 g projectile 2 days later. Rats exposed with the 100 g projectile at 9.3 m/s (x3) and 11.2 (x2) had a similar increase in the latency to platform at 2 days (and even 10 days) after exposure. No differences were seen among the groups with respect to total path length.

There was a significant decline in spontaneous exploratory activity 2 days after exposure in rats exposed repeatedly with the 50 g projectile at 9.3 and 11.2 m/s. In the first group the decline remained for 4 days and in the second it remained for 14 days.

DISCUSSION

This thesis describes a new animal model for concussion of the type seen in professional football, which is characterized by a high velocity-low mass impact to the head of a freely moving object. Preparatory surgery is not required, still, the head of the animal is reproducibly positioned. Forces are scaled from man to the rat. Data on impact speed and head acceleration, ΔV and impact duration are saved for all animals and give a low standard deviation.

The thesis also describes some aspects of the neuropathology and functional effects of the impact. There was a dose-response relationship for both. Small changes were recorded at or below impact levels of 9.3 m/s with the 50 g projectile, i.e. corresponding to the football concussion. The neuropathology consists of hemorrhages in the meninges and the brain parenchyma, abnormal accumulation of a neurofilament component in neuronal perikarya and activation of astrocytes. There was a local effect, meningeal hemorrhages, and below the point of impact, petechial hemorrhages and/or astrogliosis. The distal effect was characterized by astrogliosis and abnormal accumulation of a neurofilament component in neuronal perikarya in the cerebral cortex, hippocampus, striatum and other regions. The functional changes were cognitive deficits and reduced exploratory activity.

Immunohistochemistry

With the free floating technique, it is easier to keep whole brain sections intact, since they can be made thicker (25 μm) than paraffin embedded material (6 μm sections). The free floating technique is frequently used, since antisera diffuse from both sides into the section. The technique is combined with different freezing procedures (Hedreen et al., 1994; Yang et al., 1995; Kanayama et al., 1996). Here we used the steam evaporating from liquid nitrogen. This allows sectioning of the whole brain and preserves the morphology fairly well. In the tissue sections, cavities may arise in the boundaries between different parts of the brain, which cause difficulties in the handling of the sections. We reduced these types of problems by mounting the sections on Super Frost[®] Plus slides and drying them in air for 2 h following the microwave treatment.

Morris Water Maze

We designed a MWM paradigm that would reveal as good as possible, cognitive dysfunctions, mimicking those after concussion in football. Male Wistar rats were used, since they do spatial learning better than female rats (Brandeis et al., 1989). MWM testing is a stressful event and stress affects significantly MWM performance (Holscher 1999). A number of factors that could affect the performance were tested. The rats were always acclimatized to their new environment for at least one week before the experiment, during which the person in charge of the experiments handled the animals daily. The odor of domestic animals or any fragrance was avoided. In general, daily routines are never altered.

While repeated exposure to the 50 g projectile (matching the conditions for football concussions) reduced the exploratory activity, the cognitive performance was not significantly affected. Repeated impacts with the 100 g projectile (exceeding the conditions for football concussions) caused cognitive deficit. We have no data on exploratory activity of rats exposed with the 100 g projectile.

Comparison with other TBI models

Our system provides translational acceleration of the head in a reproducible fashion, which never leads to skull fractures. The structural damage to the brain was dose dependent and the degree of injury could easily be increased by changing the weight of the projectile and/or velocity of impact. With the conditions used, the brain damage was small compared to that inflicted with fluid percussion or weight drop. In an unpublished study, we used a 500 g projectile, i.e. a mass and impact velocity similar to that used in the weight drop model (Foda et al., 1994). The immunohistochemical results on neurofilaments and astrocytes did not differ markedly from those we obtained with a smaller mass and higher velocity. However, we observed hemorrhages in the velum interpositum (a CSF space in the roof of the third ventricle, close to the hippocampus) and dilation of the brain ventricles as seen after fluid percussion impact (Yamaki et al., 1998).

Immunohistochemistry of neurofilaments

The intermediate filaments of the neuronal cytoskeleton, the neurofilaments (NF), provide mechanical stability to the neuron. Three protein subunits assemble to form the NF (low 68 kDa, medium 160 kDa and high 200 kDa). NF68 is found predominantly in the core of assembled neurofilaments. NF-160 and NF-200 subunits are cross-linking proteins, found in the connecting branches (Gotow et al., 1994). Both have multiphosphorylated carboxy-terminal domains (Huh et al., 2002). NF is synthesized in the neuronal perikarya and transported down the axons. The carboxy terminal of NF-200 becomes heavily phosphorylated during this transport. Both phosphorylated and unphosphorylated neurofilament proteins are widely distributed through neurons and found in axons as well as dendrites (Gotow et al., 1994).

NF-200 and DAI

The definition of diffuse axonal injury (DAI) is microscopic evidence of damage to the axons, such as dissolution of microtubules, accumulation or modification of neurofilaments and proteolysis of spectrin networks (Serbest et al., 2007). DAI was initially detected with silver impregnation or Nissl staining (Marmarou et al., 1994; Huh et al., 2002; Kallakuri et al., 2003) but is now often revealed with immunostaining of the neurofilaments (Smith et al., 2003; Inglese et al., 2005). Axonal swelling, retraction balls, microglial scars, long tract degeneration and diffuse gliosis are also hallmarks of DAI (Adams et al., 1985; Simpson et al., 1985). Axonal swelling and retraction balls have been demonstrated with different antisera against either phosphorylated + unphosphorylated epitopes (N 52; Hoshino et al., 2003; Raghupathi et al., 2004; Serbest et al., 2007) or against the phosphorylated epitope of both heavy (NF-200), medium (NF-180; Ross et al., 1994) and light (NF-68; Li et al., 1998) neurofilament proteins. The phosphorylation state of NF-200 is affected by TBI (Posmantur et al., 2000).

Our aim was to use an antibody, which reacts specifically with the phosphorylated epitope NF-200 (Wang et al., 1994; Saljo et al., 2000). This antibody had been useful in earlier studies from our laboratory, since it gave an increased staining of neuronal perikarya in the injured brains, approximately 10 days after injury. The Fe3 antiserum was used with blast injured rat brains (Saljo et al., 2000) and rabbit brains after exposure of the head to rotational acceleration (Hamberger et al., 2003). Since the Fe3

antibody did not give consistent results in the present study, we tried an antibody, which recognizes the parent form of NF200 and is targeted against its carboxyl terminal at a site independent of the phosphorylation state (N52; Shaw 1986). With this antiserum we found a similar pattern as we earlier had found with Fe3, i.e. a reduced immunostaining of axons and an increased staining of neuronal perikarya 10 days after exposure.

Our results with N52 are in agreement with those of Postmantur et al (2000) 3 days after cortical impact. However, the authors also found retraction balls and fragmented axons in the corpus callosum and subcortical white matter and alterations in neurofilament immunolabeling in dendrites and cell bodies in the cortex.

The delayed NF immunoreactivity of neuronal perikarya in the cerebral cortex and the hippocampus CA1 pyramidal cells in the injured rat brains in our model is also in agreement with results after “mild injury” with lateral fluid percussion (Saatman et al., 1998). Similarly, accumulation of NF52 in neuronal perikarya in the deep cortical layers and the hippocampus is seen one week after repeated impact with fluid percussion (Kanayama et al., 1996). The increased staining of NF52 lasted up to one month (Kanayama et al., 1996). The data suggest that a single impact to the head triggers a pathological process leading to neuronal degeneration (Yaghmai et al., 1992). NF alterations may occur prior to cell death and may also contribute to transient damage or dysfunction of surviving neurons (Huh et al., 2002).

Immunohistochemistry of GFAP

Astrocytes are often activated in brain injury (Povlishock et al., 1985; Kimelberg 2005) and it seems to be a close relationship between activation of astrocytes and DAI (Norenberg 1994; Hinkle et al., 1997; Adelson et al., 2001; Onaya 2002). One day after kainic acid induced convulsions in rats, there is a significant increase in GFAP, measured quantitatively, in the rat frontal cerebral cortex and the striatum (Ding et al., 2000). GFAP levels are doubled after 3 days and reach their maximum after 7-10 days (Bodjarian et al., 1997; Ding et al., 2000; Graham et al., 2000). An increased GFAP immunoreactivity in astrocytes may be seen as early as one day after brain injury (Li et al., 1998; Graham et al., 2000) in paraffin sections incubated with polyclonal antibodies. In this study, we did not find reactive astrocytes one day after exposure, but after 7-10 days. An increase in the frequency of reactive astrocytes was seen on the impacted side after repeated impacts with the 50 g (9.3 and 11.2 m/s) and after exposure with the 100 g projectile (9.3 and 11.2 m/s). No reactive astrocytes were seen in brains exposed to a single 50 g impact at 7.4-9.3 m/s impact velocity. Reactive

astrocytes were seen in the impact site even in the absence of petechial hemorrhages, but never contralaterally. The brain regions beyond the impact site, which contained reactive astrocytes, i.e. midline regions, such as in the corpus callosum, were largely the same as observed in man after closed head injuries (Crooks et al., 1991).

The Morris Water Maze test and the hippocampus

TBI leads frequently to cognitive dysfunction in experimental studies ((Raymont et al., 2008). Athletes with a history of concussions are more likely to have future concussive injuries than those with no history. One of 15 players in college football with a concussion is likely to have additional concussions in the same playing season (Guskiewicz et al., 2003; Slobounov et al., 2007). Athletes who have experienced three or more concussions are 9 times more likely to suffer from headache, when experiencing another concussion. This may be associated with slower recovery of neurological function (Guskiewicz et al., 2003). The average concussion in professional football corresponds to a single exposure with 50 g at 9.3 m/s. Although there is a high frequency of repeated concussions in a football season (Guskiewicz et al., 2003), impairment of cognitive function is not frequently studied following repeated concussions in experimental work.

Here, the functional effects of repeated impacts with 50 g or 100 g at 9.3 and 11.2 m/s were measured with MWM. A significant impairment was seen in rats exposed to repeated impacts with 50 g projectile at 11.2 m/s or 100 g projectile at 9.3 and 11.2 m/s. Our finding that exposure to repeated impacts with the 50 g projectile at 9.3 m/s (average) had no significant effects on the MWM performance, may arise questions since the hippocampus is known as the “gateway to memory” (Kempermann 2002; Kempermann et al., 2004).

There were no differences in the swimming speed among exposed and control animals, which indicated that the “concussion” did not affect motor function.

Exploratory activity

Voluntary exploratory activity is a measure of anxiety and not associated with sensory or motor deficits after head impact (Vallee et al., 1997; Larsson et al., 2002). Voluntary exploratory activity has also been used as a measure of discomfort associated with pain (Palecek et al., 2002), distress and anxiety (Griebel et al., 1998) or adaptation to or fear of leaving a familiar place. The exploratory activity of the rats

was assessed in an open field. The computerized system measures and compares the total distance traveled during a predetermined time. Only rats exposed to repeated impacts with 50 g at 9.3 m/s and 11.2 m/s impact velocity were tested.

Limitations

No animal model simulates perfectly the conditions of sport-related concussion. There is a major difference in geometric orientation of the neuraxis and the brain anatomy differs between man and rat. Furthermore, the rat brain has no gyri and little white matter. The ethics committees require that rats are anesthetized during impact to the head. Anesthetic agents have almost invariably a neuroprotective potential (Koerner et al., 2006; Zhang et al., 2006; Head et al., 2007). The scaling of time between man and rat is not precise.

Significance and outlook

Brain injury is a considerable challenge to individuals and society. This study was designed to develop a concussion model and analyze the structural and functional effects of the impact to the head. The knowledge in the field of neuronal and glial reactions has increased dramatically over the past decades. Studies of the different kinds of brain injuries have given new hope to the prospect of structural and functional repair. Our long-term goal is to develop a treatment customized for concussion, which has both a preventive measure and a therapeutic effect. We need to explore the underlying mechanisms and biomechanics of concussion extensively. The usefulness of markers for brain injury also requires intense research. The understanding of adaptation and recovery of injured neurons may improve the neurological outcome after TBI. Factors which influence the susceptibility of NF after TBI should be identified, if possible, since prevention of NF damage may protect from degeneration. Hopefully, this thesis provides a small addition to this knowledge.

ACKNOWLEDGMENTS

I would like to sincerely express my deepest gratitude to all those who have helped and supported me during my work on this thesis, especially:

Prof. Jan-Olof Karlsson, my supervisor.

Dr Annette Säljö, my co-supervisor, for accepting me as a Ph.D. student, and for your intellectual guidance, generous support, enormous patience, and enthusiasm. Without you, I was not able to go through this journey.

Prof. Anders Hamberger, my co-supervisor for sharing your outstanding knowledge of neurobiology and excellent scientific and intellectual guidance, never-ending willingness to find the time to go through the manuscripts.

It was a great honor for me to have you and Annette as my supervisors.

Dr David Viano, NFL's representative, for your engagement and advice.

Dr Eva Jennische, for sharing your knowledge, valuable advice and practical guidance.

Mr. Berndt Svensson, for your excellent laboratory effort and your friendship.

Finally, a few words of love:

To Eric, my wonderful husband, for your encouragement, love and support throughout my Ph.D. studies.

To Alexander, my beloved son, for all the joy and happiness you have brought me.

To Fayezeah, my dear sister, for always being there for me.

The work presented in this thesis was carried out at the Institute for Biomedicine, Department for Medical Chemistry and Cell Biology, University of Gothenburg. The studies were supported by Neurotrauma Research Sweden, *NRS* AB and grants from the National Football League charities (USA).

REFERENCES

- Adams, J. H., Doyle, D., Graham, D. I., Lawrence, A. E. and McLellan, D. R. (1985). "Microscopic diffuse axonal injury in cases of head injury." *Med Sci Law* 25(4): 265-9.
- Adams, J. H. and Duchon, L. W. (1992). *Head injury in: Greenfield's Neuropathology*. London, Edward Arnold, A division of Hodder & Stoughton.
- Adelson, P. D., Jenkins, L. W., Hamilton, R. L., Robichaud, P., Tran, M. P. and Kochanek, P. M. (2001). "Histopathologic response of the immature rat to diffuse traumatic brain injury." *J Neurotrauma* 18(10): 967-76.
- Adelson, P. D., Robichaud, P., Hamilton, R. L. and Kochanek, P. M. (1996). "A model of diffuse traumatic brain injury in the immature rat." *J Neurosurg* 85(5): 877-84.
- Aubry, M., Cantu, R., Dvorak, J., Graf-Baumann, T., Johnston, K., Kelly, J., Lovell, M., McCrory, P., Meeuwisse, W. and Schamasch, P. (2002). "Summary and agreement statement of the 1st international symposium on concussion in sport, Vienna 2001." *Clin J Sport Med* 12: 6-11.
- Bleiberg, J., Cernich, A. N., Cameron, K., Sun, W., Peck, K., Ecklund, P. J., Reeves, D., Uhorchak, J., Sparling, M. B. and Warden, D. L. (2004). "Duration of cognitive impairment after sports concussion." *Neurosurgery* 54(5): 1073-78; discussion 1078-80.
- Bodjarian, N., Jamali, S., Boisset, N. and Tadie, M. (1997). "Strong expression of GFAP mRNA in rat hippocampus after a closed-head injury." *Neuroreport* 8(18): 3951-6.
- Brandeis, R., Brandys, Y. and Yehuda, S. (1989). "The use of the Morris Water Maze in the study of memory and learning." *Int J Neurosci* 48(1-2): 29-69.
- Collins, M. W., Grindel, S. H., Lovell, M. R., Dede, D. E., Moser, D. J., Phalin, B. R., Nogle, S., Wasik, M., Cordry, D., Daugherty, K. M., Sears, S. F., Nicolette, G., Indelicato, P. and McKeag, D. B. (1999). "Relationship between concussion and neuropsychological performance in college football players." *Jama* 282(10): 964-70.
- Crooks, D. A., Scholtz, C. L., Vowles, G., Greenwald, S. and Evans, S. (1991). "The glial reaction in closed head injuries." *Neuropathol Appl Neurobiol* 17(5): 407-14.
- Davies, S. J., Fitch, M. T., Memberg, S. P., Hall, A. K., Raisman, G. and Silver, J. (1997). "Regeneration of adult axons in white matter tracts of the central nervous system." *Nature* 390(6661): 680-3.
- Ding, M., Haglid, K. G. and Hamberger, A. (2000). "Quantitative immunochemistry on neuronal loss, reactive gliosis and BBB damage in cortex/striatum and hippocampus/amygdala after systemic kainic acid administration." *Neurochem Int* 36(4-5): 313-8.

- Droz, B. and Leblond, C. P. (1962). "Migration of proteins along the axons of the sciatic nerve." *Science* 137: 1047-8.
- Eichenbaum, H. (1997). "Declarative memory: insights from cognitive neurobiology." *Annu Rev Psychol* 48: 547-72.
- Fawcett, J. W. and Asher, R. A. (1999). "The glial scar and central nervous system repair." *Brain Res Bull* 49(6): 377-91.
- Foda, M. A. and Marmarou, A. (1994). "A new model of diffuse brain injury in rats. Part II: Morphological characterization." *J Neurosurg* 80(2): 301-13.
- Fujimoto, S. T., Longhi, L., Saatman, K. E. and McIntosh, T. K. (2004). "Motor and cognitive function evaluation following experimental traumatic brain injury." *Neurosci Biobehav Rev* 28(4): 365-78.
- Gavrieli, Y., Sherman, Y. and Ben-Sasson, S. A. (1992). "Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation." *J Cell Biol* 119(3): 493-501.
- Gennarelli, T. A., Thibault, L. E., Adams, J. H., Graham, D. I., Thompson, C. J. and Marcincin, R. P. (1982). "Diffuse axonal injury and traumatic coma in the primate." *Ann Neurol* 12(6): 564-74.
- Giulian, D. and Silverman, G. (1975). "Solid-state animal detection system: its application to open field activity and freezing behavior." *Physiol Behav* 14(1): 109-12.
- Goodman, J. C., Cherian, L., Bryan, R. M., Jr. and Robertson, C. S. (1994). "Lateral cortical impact injury in rats: pathologic effects of varying cortical compression and impact velocity." *J Neurotrauma* 11(5): 587-97.
- Gotow, T. and Tanaka, J. (1994). "Phosphorylation of neurofilament H subunit as related to arrangement of neurofilaments." *J Neurosci Res* 37(6): 691-713.
- Graham, D. I., Raghupathi, R., Saatman, K. E., Meaney, D. and McIntosh, T. K. (2000). "Tissue tears in the white matter after lateral fluid percussion brain injury in the rat: relevance to human brain injury." *Acta Neuropathol (Berl)* 99(2): 117-24.
- Griebel, G., Perrault, G. and Sanger, D. J. (1998). "Limited anxiolytic-like effects of non-benzodiazepine hypnotics in rodents." *J Psychopharmacol* 12(4): 356-65.
- Guskiewicz, K. M., McCrea, M., Marshall, S. W., Cantu, R. C., Randolph, C., Barr, W., Onate, J. A. and Kelly, J. P. (2003). "Cumulative effects associated with recurrent concussion in collegiate football players: the NCAA Concussion Study." *Jama* 290(19): 2549-55.

- Gutierrez, E., Huang, Y., Haglid, K., Bao, F., Hansson, H. A., Hamberger, A. and Viano, D. (2001). "A new model for diffuse brain injury by rotational acceleration: I model, gross appearance, and astrogliosis." *J Neurotrauma* 18(3): 247-57.
- Hall, E. D., Bryant, Y. D., Cho, W. and Sullivan, P. G. (2008). "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* 25(3): 235-47.
- Hamberger, A., Huang, Y. L., Zhu, Bao, F., Ding, M., Blennow, K., Olsson, A., Hansson, H. A., Viano, D. C. and Haglid, K. G. (2003). "Reditribution of neurofilaments and accumulation of beta-amyloid protein after brain injury by rotational acceleration of the head." *J Neurotrauma* 20(2): 169-78.
- Hamm, R. J., Lyeth, B. G., Jenkins, L. W., O'Dell, D. M. and Pike, B. R. (1993). "Selective cognitive impairment following traumatic brain injury in rats." *Behav Brain Res* 59(1-2): 169-73.
- Hedreen, J. C. and Koliatsos, V. E. (1994). "Phosphorylated neurofilaments in neuronal perikarya and dendrites in human brain following axonal damage." *J Neuropathol Exp Neurol* 53(6): 663-71.
- Heimer, L. (1995). *THE HUMAN BRAIN AND SPINAL CORD, Functional neuroanatomy and dissection guide*, Lennart Heimer. New York : Berlin, Springer-Vlg.
- Hinkle, D. A., Baldwin, S. A., Scheff, S. W. and Wise, P. M. (1997). "GFAP and S100beta expression in the cortex and hippocampus in response to mild cortical contusion." *J Neurotrauma* 14(10): 729-38.
- Holscher, C. (1999). "Stress impairs performance in spatial water maze learning tasks." *Behav Brain Res* 100(1-2): 225-35.
- Holsinger, T., Steffens, D. C., Phillips, C., Helms, M. J., Havlik, R. J., Breitner, J. C., Guralnik, J. M. and Plassman, B. L. (2002). "Head injury in early adulthood and the lifetime risk of depression." *Arch Gen Psychiatry* 59(1): 17-22.
- Hoshino, S., Kobayashi, S., Furukawa, T., Asakura, T. and Teramoto, A. (2003). "Multiple immunostaining methods to detect traumatic axonal injury in the rat fluid-percussion brain injury model." *Neurol Med Chir (Tokyo)* 43(4): 165-73; discussion 174.
- Huh, J. W., Laurer, H. L., Raghupathi, R., Helfaer, M. A. and Saatman, K. E. (2002). "Rapid loss and partial recovery of neurofilament immunostaining following focal brain injury in mice." *Exp Neurol* 175(1): 198-208.
- Inglese, M., Makani, S., Johnson, G., Cohen, B. A., Silver, J. A., Gonen, O. and Grossman, R. I. (2005). "Diffuse axonal injury in mild traumatic brain injury: a diffusion tensor imaging study." *J Neurosurg* 103(2): 298-303.

- Kallakuri, S., Cavanaugh, J. M., Ozaktay, A. C. and Takebayashi, T. (2003). "The effect of varying impact energy on diffuse axonal injury in the rat brain: a preliminary study." *Exp Brain Res* 148(4): 419-24.
- Kanayama, G., Takeda, M., Niigawa, H., Ikura, Y., Tamii, H., Taniguchi, N., Kudo, T., Miyamae, Y., Morihara, T. and Nishimura, T. (1996). "The effects of repetitive mild brain injury on cytoskeletal protein and behavior." *Methods Find Exp Clin Pharmacol* 18(2): 105-15.
- Kelly, J. (1997). "Sports-related recurrent brain injuries--United States." *MMWR: Morbidity & Mortality Weekly Report* 46(10): 224.
- Kelly, P. and Rosenberg, J. H. (1997). Report of the Quality Standards Subcommittee, American Academy of Neurology. Practice parameter: the management of concussion in sport (Summary statement). *Neurology*. 48: 581-585.
- Kempermann, G. (2002). "Why new neurons? Possible functions for adult hippocampal neurogenesis." *J Neurosci* 22(3): 635-8.
- Kempermann, G., Wiskott, L. and Gage, F. H. (2004). "Functional significance of adult neurogenesis." *Curr Opin Neurobiol* 14(2): 186-91.
- Kimelberg, H. K. (2005). "Astrocytic swelling in cerebral ischemia as a possible cause of injury and target for therapy." *Glia* 50(4): 389-97.
- Larsson, F., Winblad, B. and Mohammed, A. H. (2002). "Psychological stress and environmental adaptation in enriched vs. impoverished housed rats." *Pharmacol Biochem Behav* 73(1): 193-207.
- Lewen, A., Li, G. L., Nilsson, P., Olsson, Y. and Hillered, L. (1995). "Traumatic brain injury in rat produces changes of beta-amyloid precursor protein immunoreactivity." *Neuroreport* 6(2): 357-60.
- Li, R., Fujitani, N., Jia, J. T. and Kimura, H. (1998). "Immunohistochemical indicators of early brain injury: an experimental study using the fluid-percussion model in cats." *Am J Forensic Med Pathol* 19(2): 129-36.
- Lifshitz, J., Sullivan, P. G., Hovda, D. A., Wieloch, T. and McIntosh, T. K. (2004). "Mitochondrial damage and dysfunction in traumatic brain injury." *Mitochondrion* 4(5-6): 705-13.
- Lighthall, J. W. (1988). "Controlled cortical impact: a new experimental brain injury model." *J Neurotrauma* 5(1): 1-15.
- Macciocchi, S. N., Barth, J. T., Alves, W., Rimel, R. W. and Jane, J. A. (1996). "Neuropsychological functioning and recovery after mild head injury in collegiate athletes." *Neurosurgery* 39(3): 510-4.

- Makdissi, M., Collie, A., Maruff, P., Darby, D. G., Bush, A., McCrory, P. and Bennell, K. (2001). "Computerised cognitive assessment of concussed Australian Rules footballers." *Br J Sports Med* 35(5): 354-60.
- Marmarou, A., Foda, M. A., van den Brink, W., Campbell, J., Kita, H. and Demetriadou, K. (1994). "A new model of diffuse brain injury in rats. Part I: Pathophysiology and biomechanics." *J Neurosurg* 80(2): 291-300.
- McCrea, M., Kelly, J. P., Randolph, C., Cisler, R. and Berger, L. (2002). "Immediate neurocognitive effects of concussion." *Neurosurgery* 50(5): 1032-40; discussion 1040-2.
- McCrory, P., Johnston, K., Meeuwisse, W., Aubry, M., Cantu, R., Dvorak, J., Graf-Baumann, T., Kelly, J., Lovell, M. and Schamasch, P. (2005). "Summary and agreement statement of the 2nd International Conference on Concussion in Sport, Prague 2004." *Clin J Sport Med* 15(2): 48-55.
- McCrory, P., Johnston, K., Meeuwisse, W., Aubry, M., Cantu, R., Dvorak, J., Graf-Baumann, T., Kelly, J., Lovell, M. and Schamasch, P. (2005). "Summary and agreement statement of the 2nd International Conference on Concussion in Sport, Prague 2004." *Br J Sports Med* 39(4): 196-204.
- Mikulecka, A., Kubova, H. and Mares, P. (2004). "Lamotrigine does not impair motor performance and spontaneous behavior in developing rats." *Epilepsy Behav* 5(4): 464-71.
- Morris, R. (1984). "Developments of a water maze procedure for studying spatial learning in the rat." *J Neurosci Methods* 11(1): 47-60.
- Morris, R. G., Garrud, P., Rawlins, J. N. and O'Keefe, J. (1982). "Place navigation impaired in rats with hippocampal lesions." *Nature* 297(5868): 681-3.
- Mortimer, J. A., van Duijn, C. M., Chandra, V., Fratiglioni, L., Graves, A. B., Heyman, A., Jorm, A. F., Kokmen, E., Kondo, K., Rocca, W. A. and et al., (1991). "Head trauma as a risk factor for Alzheimer's disease: a collaborative re-analysis of case-control studies. EURODEM Risk Factors Research Group." *Int J Epidemiol* 20 Suppl 2: S28-35.
- Nilsson, B., Ponten, U. and Voigt, G. (1977). "Experimental head injury in the rat. Part 1: Mechanics, pathophysiology, and morphology in an impact acceleration trauma model." *J Neurosurg* 47(2): 241-51.
- Norenberg, M. D. (1994). "Astrocyte responses to CNS injury." *J Neuropathol Exp Neurol* 53(3): 213-20.
- O'Connor, C., Heath, D. L., Cernak, I., Nimmo, A. J. and Vink, R. (2003). "Effects of daily versus weekly testing and pre-training on the assessment of neurologic impairment following diffuse traumatic brain injury in rats." *J Neurotrauma* 20(10): 985-93.

- Ommaya, A. K., Yarnell, P., Hirsch, A. and Harris, E. H. (1967). Scaling of experimental data on cerebral concussion in sub-human primates to concussion threshold for man. 11th Stapp Car Crash Conference, SAE 670906, Society of Automotive Engineers, Warrendale, PA, 73-80, 1.
- Onaya, M. (2002). "Neuropathological investigation of cerebral white matter lesions caused by closed head injury." *Neuropathology* 22(4): 243-51.
- Palecek, J., Paleckova, V. and Willis, W. D. (2002). "The roles of pathways in the spinal cord lateral and dorsal funiculi in signaling nociceptive somatic and visceral stimuli in rats." *Pain* 96(3): 297-307.
- Paxinos, G. and Watson, C. (1982). *The rat brain*, Academic press, a subsidiary of Harcourt Brace Jovanovich.
- Pellman, E. J., Lovell, M. R., Viano, D. C., Casson, I. R. and Tucker, A. M. (2004). "Concussion in professional football: neuropsychological testing--part 6." *Neurosurgery* 55(6): 1290-303; discussion 1303-5.
- Pellman, E. J., Powell, J. W., Viano, D. C., Casson, I. R., Tucker, A. M., Feuer, H., Lovell, M., Waeckerle, J. F. and Robertson, D. W. (2004). "Concussion in professional football: epidemiological features of game injuries and review of the literature--part 3." *Neurosurgery* 54(1): 81-94; discussion 94-6.
- Pellman, E. J., Viano, D. C., Casson, I. R., Arfken, C. and Feuer, H. (2005). "Concussion in professional football: players returning to the same game--part 7." *Neurosurgery* 56(1): 79-90; discussion 90-2.
- Pellman, E. J., Viano, D. C., Tucker, A. M. and Casson, I. R. (2003). "Concussion in professional football: location and direction of helmet impacts-Part 2." *Neurosurgery* 53(6): 1328-40; discussion 1340-1.
- Pellman, E. J., Viano, D. C., Tucker, A. M., Casson, I. R. and Waeckerle, J. F. (2003). "Concussion in professional football: reconstruction of game impacts and injuries." *Neurosurgery* 53(4): 799-814.
- Plassman, B. L., Havlik, R. J., Steffens, D. C., Helms, M. J., Newman, T. N., Drosdick, D., Phillips, C., Gau, B. A., Welsh-Bohmer, K. A., Burke, J. R., Guralnik, J. M. and Breitner, J. C. (2000). "Documented head injury in early adulthood and risk of Alzheimer's disease and other dementias." *Neurology* 55(8): 1158-66.
- Ponsford, J., Draper, K. and Schonberger, M. (2008). "Functional outcome 10 years after traumatic brain injury: Its relationship with demographic, injury severity, and cognitive and emotional status." *J Int Neuropsychol Soc* 14(2): 233-42.

- Posmantur, R. M., Newcomb, J. K., Kampfl, A. and Hayes, R. L. (2000). "Light and confocal microscopic studies of evolutionary changes in neurofilament proteins following cortical impact injury in the rat." *Exp Neurol* 161(1): 15-26.
- Povlishock, J. T. and Becker, D. P. (1985). "Fate of reactive axonal swellings induced by head injury." *Lab Invest* 52(5): 540-52.
- Powell, J. W. and Barber-Foss, K. D. (1999). "Traumatic brain injury in high school athletes." *Jama* 282(10): 958-63.
- Raghupathi, R., Mehr, M. F., Helfaer, M. A. and Margulies, S. S. (2004). "Traumatic axonal injury is exacerbated following repetitive closed head injury in the neonatal pig." *J Neurotrauma* 21(3): 307-16.
- Raivich, G., Bohatschek, M., Kloss, C. U., Werner, A., Jones, L. L. and Kreutzberg, G. W. (1999). "Neuroglial activation repertoire in the injured brain: graded response, molecular mechanisms and cues to physiological function." *Brain Res Brain Res Rev* 30(1): 77-105.
- Raymont, V., Greathouse, A., Reding, K., Lipsky, R., Salazar, A. and Grafman, J. (2008). "Demographic, structural and genetic predictors of late cognitive decline after penetrating head injury." *Brain* 131(Pt 2): 543-58.
- Reire, P. L. (1986). "Gliosis following CNS injury: the anatomy of astrocytic scars and their influences on axonal elongation." *Cell Biol Pathol Astrocytes*. Orlando, FL, Academic press,: 263-324.
- Ross, D. T., Meaney, D. F., Sabol, M. K., Smith, D. H. and Gennarelli, T. A. (1994). "Distribution of forebrain diffuse axonal injury following inertial closed head injury in miniature swine." *Exp Neurol* 126(2): 291-9.
- Saatman, K. E., Graham, D. I. and McIntosh, T. K. (1998). "The neuronal cytoskeleton is at risk after mild and moderate brain injury." *J Neurotrauma* 15(12): 1047-58.
- Saljo, A., Bao, F., Haglid, K. G. and Hansson, H. A. (2000). "Blast exposure causes redistribution of phosphorylated neurofilament subunits in neurons of the adult rat brain." *J Neurotrauma* 17(8): 719-26.
- Sallis, R. E. and Jones, K. (2000). "Prevalence of headaches in football players." *Med Sci Sports Exerc* 32(11): 1820-4.
- Serbest, G., Burkhardt, M. F., Siman, R., Raghupathi, R. and Saatman, K. E. (2007). "Temporal profiles of cytoskeletal protein loss following traumatic axonal injury in mice." *Neurochem Res* 32(12): 2006-14.
- Shaw, G. (1986). "Neurofilaments: abundant but mysterious neuronal structures." *Bioessays* 4(4): 161-6.

- Simpson, R. H., Berson, D. S. and Shapiro, H. A. (1985). "The diagnosis of diffuse axonal injury in routine autopsy practice." *Forensic Sci Int* 27(4): 229-35.
- Slobounov, S., Slobounov, E., Sebastianelli, W., Cao, C. and Newell, K. (2007). "Differential rate of recovery in athletes after first and second concussion episodes." *Neurosurgery* 61(2): 338-44; discussion 344.
- Smith, D. H., Meaney, D. F. and Shull, W. H. (2003). "Diffuse axonal injury in head trauma." *J Head Trauma Rehabil* 18(4): 307-16.
- Smith, D. M. and Mizumori, S. J. (2006). "Hippocampal place cells, context, and episodic memory." *Hippocampus* 16(9): 716-29.
- Stoll, G., Jander, S. and Schroeter, M. (1998). "Inflammation and glial responses in ischemic brain lesions." *Prog Neurobiol* 56(2): 149-71.
- Sullivan, H. G., Martinez, J., Becker, D. P., Miller, J. D., Griffith, R. and Wist, A. O. (1976). "Fluid-percussion model of mechanical brain injury in the cat." *J Neurosurg* 45(5): 521-34.
- Valentinuzzi, V. S., Menna-Barreto, L. and Xavier, G. F. (2004). "Effect of circadian phase on performance of rats in the Morris water maze task." *J Biol Rhythms* 19(4): 312-24.
- Vallee, M., Mayo, W., Dellu, F., Le Moal, M., Simon, H. and Maccari, S. (1997). "Prenatal stress induces high anxiety and postnatal handling induces low anxiety in adult offspring: correlation with stress-induced corticosterone secretion." *J Neurosci* 17(7): 2626-36.
- Viano, D. C. and Pellman, E. J. (2005). "Concussion in professional football: biomechanics of the striking player--part 8." *Neurosurgery* 56(2): 266-80; discussion 266-80.
- Wang, S., Hamberger, A., Yang, Q. and Haglid, K. G. (1994). "Changes in neurofilament protein NF-L and NF-H immunoreactivity following kainic acid-induced seizures." *J Neurochem* 62(2): 739-48.
- Yaghamai, A. and Povlishock, J. (1992). "Traumatically induced reactive change as visualized through the use of monoclonal antibodies targeted to neurofilament subunits." *J Neuropathol Exp Neurol* 51(2): 158-76.
- Yamaki, T., Murakami, N., Iwamoto, Y., Sakakibara, T., Kobori, N., Ueda, S., Uwahodo, Y. and Kikuchi, T. (1998). "Cognitive dysfunction and histological findings in rats with chronic-stage contusion and diffuse axonal injury." *Brain Res Brain Res Protoc* 3(1): 100-6.
- Yang, Q., Hamberger, A., Hyden, H., Wang, S., Stigbrand, T. and Haglid, K. G. (1995). "S-100 beta has a neuronal localisation in the rat hindbrain revealed by an antigen retrieval method." *Brain Res* 696(1-2): 49-61.

- Yu, A. C., Lee, Y. L. and Eng, L. F. (1993). "Astrogliosis in culture: I. The model and the effect of antisense oligonucleotides on glial fibrillary acidic protein synthesis." *J Neurosci Res* 34(3): 295-303.
- Zhu, H., Wang, Z. Y. and Hansson, H. A. (2003). "Visualization of proliferating cells in the adult mammalian brain with the aid of ribonucleotide reductase." *Brain Res* 977(2): 180-9.