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Behavioral and Histological Effects of Traumatic Brain Injury on Alzheimer's Disease Transgenic Mice

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Behavioral and Histological Effects of Traumatic Brain Injury on Alzheimer's Disease Transgenic Mice

by

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Table of Contents

List of Figures

Abstract

The main objective of this study was to elucidate the possible mechanistic link between traumatic brain injury (TBI) and Alzheimer's disease (AD) using an animal model. We examined behavioral and histological effects of TBI in pre-symptomatic AD-transgenic mice (C57B6/SJL/SwissWebster/B6D2F1). In previous studies, these mice displayed AD-like behavioral deficits by 15-17 months of age and AD-like neuropathology as early as six months of age. To clarify the effects of TBI on these mice, the present study began when they were about three months of age and the study ended when they were about five months of age. As a control, non-transgenic (NT) mice were also evaluated in this study. To assess behavioral changes following TBI, all mice were subjected to 14 days of pre-TBI training of a spatial memory task, the radial arm water maze (RAWM). After training, there were no performance differences between AD-transgenic mice and NT mice. Then, half of the AD-transgenic mice, as well as half of the NT mice, received an experimental TBI at the right parietal cortex using a pneumatic impactor. The other half of these mice received sham surgery. At two, four, and six weeks after surgery, all mice were tested in the same water maze task and the numbers of errors were recorded. AD-transgenic mice with TBI made significantly more errors than AD-transgenic mice without TBI and NT mice regardless of TBI. Furthermore, deficits were observed at both two and six weeks after TBI surgery. To assess histological changes following TBI, we used a monoclonal antibody against beta-amyloid (Aβ) to

detect AD-like plaques and an antibody against NeuN to evaluate the total neuronal loss. There were no clear group differences in terms of the Aβ expression pattern, although one AD-transgenic mouse with TBI showed AD-like Aβ plaques throughout the entire cortex and hippocampus. These results suggest that TBI precipitated behavioral deficits in a spatial memory task in pre-symptomatic AD-transgenic mice, but not control mice. Further studies are warranted for histological effects of TBI.

Chapter 1:

Introduction

The main objective of this project was to elucidate the possible link between traumatic brain injury (TBI) and Alzheimer's disease (AD). The two hallmarks of AD neuropathology include neurofibrillary tangles comprised of tau protein and accumulated beta amyloid (Aβ) protein-containing plaques (Alzheimer's Association, 2010). Recent research shows that AD-like neuropathological characteristics can be observed following TBI (Johnson, Stewart, & Smith, 2010). Based on these findings, the main hypothesis of this project was that TBI precipitates AD.

Although Aβ plaques have been detected postmortem in people who had experienced TBI (Gorrie, Oakes, Duflou, Blumbergs, & Waite, 2002; Johnson et al., 2010; Mortimer, French, Hutton, & Schuman, 1985; Roberts, Allsop, & Bruton, 1990; Uryu et al., 2007), the exact mechanistic link between TBI and AD remains unexplored. In order to clarify the relationship, an animal model is essential. Specifically, using transgenic mice that have been engineered to develop AD-like behavior and pathology later in life will be effective to study the behavioral and pathological effects of TBI. Therefore, this study had two specific aims.

Aim 1: To examine the behavioral effects of TBI on pre-symptomatic AD-transgenic mice.

Aim 2: To examine the histological effects of TBI on pre-symptomatic AD-transgenic mice.

For Aim 1, I expected that AD-transgenic mice after TBI would show more cognitive deficits than control animals, such as non-transgenic (NT) mice with TBI and AD-transgenic mice without TBI. For Aim 2, I expected that AD-transgenic mice after TBI would show more severe histological effects (e.g., increased lesion volume, advanced AD-like pathology) than control animals.

Traumatic Brain Injury

TBI, or an external impact on the head that causes physical and functional damage to the brain, accounts for approximately one third of all injury related deaths and 1.5 million emergency room visits and hospitalizations per year in the United States (Coronado et al., 2010). Over five million people are living with a disability related to TBI (Langlois, Rutland-Brown, & Wald, 2006). People in different age groups can experience TBI from a variety of causes. For instance, young children and elderly can have TBI by falling, whereas sports-related injuries are common in adolescents. Injuries from automobile accidents can also occur across all age groups. Furthermore, the National Academy of Sciences (2008) reports that TBI accounts for 22% of all soldier deaths; 59% of soldiers who were exposed to combat blasts and evaluated for brain damage suffered from TBI.

TBI can cause multiple types of brain damage, including the initial mechanical impact on the outer part of the brain, the cerebral cortex and underlying white matter. The initial impact can also damage blood vessels in the brain surface, causing hemorrhaging which can then lead to the formation of a cerebral hematoma. This pool, or pocket, of

blood not only disrupts the normal blood flow of the cortex, but also applies pressure to other brain structures and/or other blood vessels, furthering damage to subcortical regions (Coronado et al., 2010).

Sufferers of TBI may experience various functional changes including disturbances in sensation, e.g. dizziness and vertigo (Maskell, Chiarelli, & Isles, 2006), working memory (Christodoulou et al., 2001), and language (Yang, Fuller, Khodaparast, & Krawczyk, 2010). The long term effects of TBI can vary; some people's fates are much more dramatic and extensive than others. Following even a relatively mild TBI, returned soldiers report several symptoms including chronic headaches, back pain, decreased concentration, irritability, and fatigue (Hoge et al., 2008). More serious issues following TBI include post-traumatic stress disorder, dementia pugilistica, also known as chronic traumatic encephalopathy and punch-drunk syndrome, and possibly AD (DeKosky, Ikonomovic, & Gandy, 2010).

Alzheimer's Disease

According to the 2010 Alzheimer's Association report, AD accounts for approximately 60-80% of all dementia cases, overwhelmingly more than other dementias, such as vascular dementia, Lewy body dementia, and frontotemporal dementia. One in every eight people age 65 and older suffers from AD, and by the year 2050, a person will develop AD every 33 seconds (Alzheimer's Association, 2010).

One of the most significant mental and cognitive characteristics of AD is progressive and severe disturbance in the formation of new memory, declarative memory in particular (Carlesimo, Perri, & Caltagirone, 2011; Dubois et al., 2007; Terry & Davies, 1980). It usually starts with subtle forgetfulness. As the disease progresses, significant

loss of immediate memory becomes evident and eventually all mental and cognitive abilities are irreversibly impaired (Terry & Davies, 1980).

The hallmarks of pathological changes associated with AD are neurofibrillary tangles and Aβ plaques. Aβ is a protein fragment cleaved from an amyloid precursor protein (APP). While Aβ in a healthy brain contains 40 amino acids, Aβ in the brain of most AD patients is the 42-residue form which accumulates and forms hard, insoluble plaques in the extracellular spaces, eventually causing widespread atrophy (Selkoe, 2001).These plaques are most common in the hippocampal formation and related limbic structures, as well as the cerebral cortex, areas known to be involved in the function of memory formation (Sperling et al., 2010).

A Possible Relationship Between Brain Injury and Alzheimer's Disease

There are previous studies implicating a possible connection between TBI and AD. Mortimer, French, Hutton, and Schuman (1985) evaluated the frequency of head injuries for 78 patients with dementia of the Alzheimer type. Although these patients could not necessarily confirm details related to the injuries by themselves due to dementia, family members or surrogates of the patients reported significantly more head injuries (20.6%) than the other non-AD patients (5.3%). There were 30 patients who passed away during the study, 16 of which had autopsies performed. Fourteen of these deceased participants were clinically diagnosed as having AD (Mortimer et al., 1985). This study triggered a search of the relationship between TBI and a clinical diagnosis of AD.

In terms of the connection between the AD neuropathology and TBI, one of the first studies was done by Roberts, Allsop, and Bruton (1990), who reexamined brains of

boxers who had been diagnosed with dementia pugilistica. The original report of these patients had only mentioned the presence of neurofibrillary tangles (Corsellis, Bruton, & Freeman-Browne, 1973), which was then not regarded as AD neuropathology. Using immunocytochemical methods, Roberts et al. (1990) were able to show these brains also had extensive Aβ plaques. Roberts, Gentleman, Lynch, and Graham (1991) further showed AD-like pathology could appear almost immediately after TBI, including Aβ deposits in the cortex of six of 16 patients (age range 10-63 years) who died within 6-18 days after TBI. The same authors later conducted a more extensive study with 152 patients (age range 8 weeks-85 years) whose survival times ranged between four hours and 2.5 years post TBI and confirmed Aβ deposits in 30% of the patients (Roberts et. al, 1994).

Similar findings about AD-like pathology after TBI have been reported by other authors as well (Huber, Gabbert, Kelemen, & Cervod-Navarro, 1993; Smith, Chen, Iwata, & Graham, 2003). Moreover, some studies focused specifically on the survival time of the TBI victims, showing AD-like pathology whether the person suffered a fatal TBI (Gentleman et al., 1997), passed away days later (Horsburgh et al., 2000), lived for an extended period of time following TBI (Chen, Johnson, Uryu, Trojanowski, & Smith, 2009), or who were still alive (DeKosky et al., 2007; Ikonomovic et al., 2004).

Furthermore, studies showed that the development of AD-like pathology could occur not only in adult TBI victims, but also young children after TBI. Roberts et al. (1994) had reported that no victims under age 10 showed Aβ deposits. However, Gorrie, Oakes, DuFlou, Blumbergs, and Waite (2002) examined 32 children (ages 3 months - 16 years) who died following motor vehicle collisions. When their brains were examined, 14

cases showed β-APP immunoreactivity in parasagittal white matter (12/14), corpus callosum (11/14) and brainstem (10/14) (Gorrie et al., 2002). Collectively, these data show that AD-like pathology occurs independent of the cause of TBI, the survival time after TBI, and possibly the age of the victim of TBI.

Animal Studies on the Relationship Between TBI and AD

A variety of animal models have been developed to investigate the relationship between TBI and AD, using swine, rats and transgenic mice (Johnson et al., 2010). Histologically, swine were the first animal models to replicate human Aβ pathology (Smith et al., 1999). Swine are typically used in a rotational acceleration model to produce diffused axonal damage which causes an initial increase in Aβ plaques (Chen et al., 2004). However, the AD-like pathology did not worsen with time. Similarly, the traditional, commonly used NT rat models show an increase in APP immunoreactivity after TBI, but no Aβ deposits or plaques were found in any subjects (Lewen, Li, Nilsson, Olsson, & Hillered, 1995; Pierce, Trojanowski, Graham, Smith, & McIntosh, 1996).

Therefore, transgenic mouse models, specifically those engineered to develop human familial AD-like pathology, have been used to further study the effects of TBI in relation to AD in rodent models. Earlier studies showed that following TBI, APP-transgenic mice had increased levels of Aβ peptide within regional brain tissues (using the enzyme-linked immunosorbent assays methods) and showed neuronal loss in the hippocampus and the cortex (Murai et al., 1998; Smith et al., 1998). However, no Aβ plaques were detected using the immunhistochemistry methods. In accordance with these studies, other research groups also found that TBI triggered an increase in Aβ peptide levels in the hippocampus in AD-transgenic mice, whereas no obvious plaques were

detected (Abrahamson et al., 2006; Abrahamson, Ikonomovic, Dixon, & DeKosky, 2009; Hartman et al., 2002; Loane et al., 2009).

Researchers have examined the cognitive and behavioral effects of TBI on AD-transgenic mice. However, the results have been rather inconsistent. For instance, Smith et al. (1998) trained mice in the Morris water maze, administered TBI, and retested the mice one week later. In the task, subject animals in a water pool are required to remember the location of a hidden platform. AD-transgenic mice with TBI showed a significant impairment compared to NT mice with TBI. However, there was also a study showing that TBI caused a deficit in the same memory task for both AD-transgenic and NT mice (Murai et al., 1998). There are also studies showing that behavioral deficits after TBI could be ameliorated by various pharmacological therapies (Loane et al., 2009; Abrahamson et al., 2009). For example, in Loane et al. (2009), following TBI, mice were treated with DAPT (a pharmacologic inhibitor of APP-related enzyme activity). The AD-transgenic mice treated with DAPT after TBI performed as well as sham controls on the Morris swim task.

Rationale

The possible relationship between TBI and AD remains unclear. Thus far the histological and behavioral results have been inconsistent and cannot demonstrate the apparent downstream cell atrophy and death mechanistic link between TBI and AD. Therefore, an animal model that potentially develops AD-like pathology combined with an established TBI method is necessary to examine this relationship.

Chapter 2:

Design and Methods

Basic Design of Study

In the present study, pre-symptomatic AD-transgenic mice and NT control mice were subjected to 1) pre-TBI behavioral testing, 2) the TBI procedure, 3) post-TBI behavioral testing, 4) neurological assessment of sensorimotor functions, and lastly 5) euthanasia for the histological analysis of the brain tissues.

The basic flow of the present study is presented in Figure 1.

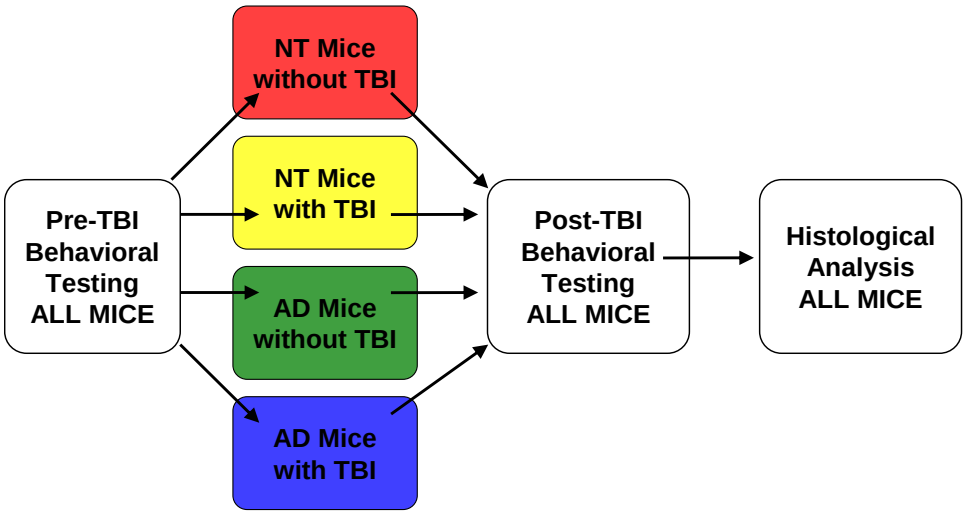

Figure 1: Basic design of the study.

While the AD-transgenic mouse model for the present study had not yet been investigated in a TBI study, AD-related histological pathology and behavioral performance of these mice of both single (APP) and double mutations (APP and

APP+PS1[1]) have been previously studied. Thus, Holcomb et al., (1998) showed that the development of Aβ deposits and plaques in the APP-PS1 mice was accelerated as early as 3-6 months of age. In APP mice, the same pathology was evident by 10-13 months of age (Hsiao et al., 1996). As for cognitive and behavioral performance, Holcomb et al. (1999) found that, compared to APP mice and NT-mice, APP-PS1 mice started to show behavioral deficits by six months of age in a Y-maze task, but not in the Morris water maze task. Repeated testing showed little change in these varying behavioral deficits at nine months of age, despite the brains of these animals exhibiting Aβ deposits and plaques (Holcomb et al., 1999).

Arendash et al. (2001) also showed that both APP and APP-PS1 mice at 15-17 months of age made significantly more errors compared to controls in the Morris swim maze, as well as radial water maze (RAWM). Upon the histological analyses of these mice, researchers showed a positive correlation between Aβ deposits and plaques and impaired behavioral performance (Gordon et al., 2001). In the present study, I used APP mice that were still pre-symptomatic in terms of histopathology and behavioral performance.

The method of TBI exposure also requires fine control and reliable reproducibility. Both the fluid percussion and the Controlled Cortical Impactor (CCI) methods were commonly used in many previous animal TBI studies. In particular, the CCI method allows the researcher to control the velocity, depth, duration, and angle of the TBI (Hayashi et al., 2009). By manipulating these measurements, Yu et al. (2009) showed the severity of TBI (mild, moderate, or severe) significantly relates to cognitive

[1] The presenilin-1(PS1) gene, expressed primarily in cerebellar and hippocampal neurons, is also associated with the earlier onset of Alzheimer's disease (Liu et al., 2009; Shen et al. 1997).

and behavioral impairments. In the present study, this CCI method was used to produce head trauma.

Methods

Experiment 1: Behavioral Examination. The goal of Experiment 1 was to examine the behavioral/cognitive effects of TBI on AD-transgenic mice using the RAWM test. The independent variables were genotype (AD-transgenic or NT) and TBI exposure (TBI or Sham). The dependent variable was the number of performance errors made during each trial.

Subjects. A total of 38 three-month-old mice (19 AD-transgenic, 19 NT) originally comprised the subject pool. Following the pre-TBI behavioral testing, the mice were divided into four groups: AD-transgenic mice exposed to TBI (AD-TBI), AD-transgenic mice without exposure to TBI (AD-Sham), NT mice exposed to TBI (NT-TBI), and NT mice without TBI (NT-Sham). Each mouse was housed in an individual cage in the USF Psychology Animal Facility, where water and food were accessible and a 12 hour light-dark cycle was maintained. All methods were carried out according to the NIH Guide for the Care and Use of Laboratory Animals under the approval of the Institutional Animal Care & Use Committee, University of South Florida.

Transgenic and Non-transgenic Subjects. The subjects were obtained through pre-determined breeder pairs, combining mutant Tg 2576 APP mice with mutant PS1 line 5.1 Tg mice, which will generate NT, APP, APP+PS1, and PS1 transgenic offspring with a mixed background of (C57B6/SJL/SwissWebster/B6D2F1). Upon weaning, subjects were genotyped by Southern blot analysis. APP and NT mice were used in this study.

Apparatus. The apparatus was a black circular pool (100 cm diameter), in which an aluminum insert was placed to form a RAWM (Fig. 2). In the apparatus, six arms (32 cm length and 19 cm width) radiated from the central circular region (36 cm diameter). The clear round platform (9 cm diameter) was placed in one of the arms at 1.5 cm beneath the surface of the water. Surrounding the pool, a visual cue was placed at each end of the radial arms, including an inflatable flamingo, a bouquet of silk flowers, a bean bag pumpkin, a plush flower, and a beach ball. These visual cues were placed at random heights and proximity to the pool and aligned with the center of each arm. The experimenter remained in the room during each session and served as one of the visual cues, standing at the end of one non-goal arm.

Figure 2: Photograph of the RAWM and Visual Cues.

Procedure.

Pre-TBI Behavioral Training and Testing. Each session consisted of four acquisition trials (T1–T4), followed by a 30-min delay interval and then a retention trial (T5). For each session, an escape platform was placed at the end of the goal arm, which was different between sessions. At the beginning of each trial, the subject was placed at the end of one of the remaining five non-goal arms. The mouse was positioned facing the wall, away from the center. Each trial lasted 60 seconds, during which an animal was

allowed to swim in order to find the platform. The latency to locate the submerged platform and the number of entries to the non-goal arms were recorded as errors. The error occurred when the subject's full body length entered into an incorrect arm, including the goal arm if the platform was not found. The subject was allowed to swim to the end of the incorrect arm, and then gently guided back to the starting location after every error. Once the subject found the platform, a 30 second resting period on the platform was permitted before beginning the next trial for T1-T4. If the subject failed to find the platform within 60 seconds, it was guided to the platform and allowed a 30 second resting period. After the completion of T4, the mouse was removed from the pool, dried with a towel, and returned to its cage for 30 minutes. The procedures for T5 were identical to T1-T4. Only one session per day was performed. The Pre-TBI testing sessions continued for a total of 14 days until all mice performed at an average of two or fewer errors across T4 and T5. The experimenter remained unaware of the animals' genotypes throughout all of the behavioral testing.

TBI Procedure. Once animals reached the criterion for the pre-TBI behavioral testing, they were deeply anesthetized using the intraperitoneal injection of a combination of ketamine (100mg/Kg) and xylazine (10mg/Kg). Mice were then placed in a stereotaxic apparatus device (David Kopf Instruments) attached to a CCI (Pittsburgh Precision Instruments, Inc.). The target of the TBI was over the right frontoparietal cortex, where the skull was exposed and a burr hole (4 mm in diameter) was drilled. The metal impactor rod (3 mm in diameter) was angled 15° to the vertical to be perpendicular to the tangential plane of the brain curvature at the impact surface. Using a pneumatic system, the impactor rod collided with the brain at a velocity of 6.0 m/s reaching a depth 2.0 mm

below the dura mater and remained for five seconds. The velocity and duration were verified by a linear variable displacement transducer (Macrosensors, Pennsauken, NJ), which was connected to the impactor. After the procedure, the incision was sutured using nonabsorbable suture material (nylon Ethilon®). Animals for sham injury surgeries underwent anesthesia, scalp incision, craniectomy, and suturing. All CCI brain injuries and sham treatments were conducted during the light phase by an investigator unaware of the animals' genotypes.

Post-TBI Behavioral Testing. Three post-TBI testing sessions were conducted two, four, and six weeks after the TBI procedure. The procedures were the same as the pre-TBI testing, except that the post-TBI testing consisted of four consecutive sessions regardless of the behavioral performance. The experimenter remained unaware of the animals' genotypes and surgery types throughout the behavioral testing.

Neurological Assessment. Directly following the completion of three post-TBI testing sessions, neurological functioning for all groups was assessed using the following tasks: balance beam, string agility, and the visual cliff.

The balance beam task was used to examine subjects' motor coordination and balance. The apparatus was constructed of a 50 cm long x 1 cm wide rod connecting two escape platforms (10cm x 14cm), all of which stood 45 cm above a cushioned table surface. Each mouse was placed in the middle of the balance beam, perpendicular to the balance beam. The amount of time before falling was recorded, with a maximum of 60 seconds. If the animal was able to walk on the balance beam, to the escape platform, "60 seconds" was recorded for the animal.

The string agility task was used to examine subjects' grip capacity, strength and agility. The apparatus included the same escape platforms of the balance beam, but a taut string connects the two platforms instead. The animal was placed in the middle of the two platforms, and allowed to grip the string with its front two paws. Once both paws were on the string, the experimenter released the animal. During a 60-second trial, the success or failure of the mouse to remain suspended from the string was recorded as a "1" for success and a "0" for a failure.

The visual cliff task examined the visual sensitivity to depth, and more importantly the perception of possible danger of falling. The apparatus was a clear plexiglas "floor" that was placed over a black and white checkered "visual cliff". The visual cliff was located 7.5 cm lower than the clear floor. A "1" was assigned if the animal showed hesitation toward walking over the visual cliff, and a "0" if the animal showed no hesitation at all.

Analysis. All genotype/TBI groups were assessed statistically using IBM SPSS Statistics software. For the multiple post-TBI RAWM sessions, a 2 x 2 ANOVA was conducted with genotype (AD, NT) and TBI exposure (TBI, Sham) as the between-subjects factors. The errors of T4 and T5 were the dependent variables focused on to measure behavioral deficit. For the neurological assessment tasks, a 2 x 2 ANOVA (genotype x TBI exposure) was used to measure any group differences in the balance beam and visual cliff tasks; a chi square analysis was used to measure any group differences on the string agility task. Upon observing any significant differences between groups, subsequent t-tests were also conducted. All group comparisons having $p < .05$ were considered significant.

Experiment 2: Histological Examination. The goal of Experiment 2 was to examine the histological effects of TBI on AD-transgenic mice. The same animals used in Experiment 1 were used for Experiment 2. Following the completion of all behavioral testing, the animals were euthanatized and the brain tissues were processed to examine the extent of cerebral damage and detection of AD-like neuropathology.

Perfusion. The subjects were sacrificed the day after all behavioral testing and neurological assessments were complete, with an overdose of Ketamine hydrochloride and Xylazine. The animals were perfused transcardially with 0.9% saline followed by ice-cold 4.0% paraformaldehyde in 0.1 M phosphate buffer solution (PBS). The brains were harvested and placed in 4.0% paraformaldehyde in PBS for overnight, followed by 30% Sucrose in PBS for at least 24 hours. The brains were frozen and cut in transverse sections at 35-40 μm on a sliding microtome. Some tissues were processed for immunohistochemistrical analyses (reactivity of Aβ and NeuN, specifically), while others were mounted on slides and stained with cresyl violet. All slides were dehydrated with an ethanol series, cleared with Citrisolv (Fisher Scientific, Fair Lawn, N.J.) and coverslipped with Permount (Fisher Scientific, Fair Lawn, N.J.).

Lesion Reconstruction. Lesion sites and volumes were microscopically analyzed and traced using a camera lucida method. Cortical, hippocampal, and overall lesion volumes were determined by calculating the total volumes of neuronal loss in the injured hemisphere compared to the contralateral side.

Immunohistochemistry. To detect AD-like pathology, the immunohistochemical methods with an antibody against Aβ were used. An antibody against NeuN was also used to investigate neuronal loss. In addition, antibodies against Ki67 and Nestin were

used to investigate the level of neurogenesis. Tissues were washed in 0.1 M PBS and then incubated in a solution of 0.3% Triton X-100 in PBS with either biotin labeled Aβ 17-24 (4G8) monoclonal antibody (1:1,000, Covance Research Products, Emeryville, CA) or biotin labeled anti-NeuN (clone A60) monoclonal antibody (1:10,000, Millipore, Temecula, CA) overnight at 4° C. Other tissues were incubated in a solution of 0.3% Triton X-100 in PBS with either rabbit polyclonal to Ki67 - proliferation marker ab15580 (1:10,000, Abcam, Cambridge, MA), monoclonal Ki67 antibody (1:10,000, Novus Biologicals, Littleton, CO), rabbit polyclonal to Nestin ab7659 (1:10,000, Abcam, Cambridge, MA), or monoclonal [2Q178] to Nestin ab6142 (1:10,000, Abcam, Cambridge, MA) overnight at 4° C. The tissues were washed in 0.1 M PBS and processed according to the manufacturer protocol of avidin biotin complex method (ABC Elite Kit, PK-6100, Vector) and visualized with 0.025% solution of 3’3 diaminobenzadine and hydrogen peroxide. The processed tissues were mounted on subbed, glass slides, dehydrated in an ethanol series, cleared, and coverslipped.

Tissue Examination. Tissues were examined using a macroscope (Wild M420 and Nikon SMZ 1500) and a microscope (Nikon Microphot FX). Images were captured using CCD/digital cameras (Spot Insight QE or Nikon DXM1200) mounted on either macro- or microscopes.

Chapter 3:

Results

Experiment 1: Behavioral Examination

Pre-TBI RAWM Performance. Before TBI was performed, 19 AD-transgenic and 19 NT mice were trained to perform in the RAWM. All mice performed at an average of two or fewer errors across T4 and T5 by the end of 14th day. Animals in each genotype were then divided into the TBI and Sham lesion groups to counterbalance learning performance. Figure 3 shows the mean numbers of errors for NT-Sham (n=9), NT-TBI (n=10), AD-Sham (n=9), and AD-TBI (n=10) mice. A 2 x 2 Factorial ANOVA was conducted with genotype (NT, AD) and planned surgical treatment (TBI, Sham) for T4 and T5. For T4, There was no significant effect for genotype, $F(1,37)=0.019$, $p=.890$, treatment, $F(1,37)=0.024$, $p=.877$, or interaction of genotype by treatment, $F(1,37)=0.154$, $p=.697$. For T5, there was also no significant effect for genotype, $F(1,37)=0.154$, $p=.697$, treatment, $F(1,37)=0.292$, $p=.592$, or interaction of genotype by treatment, $F(1,37)=0.032$, $p=.858$.

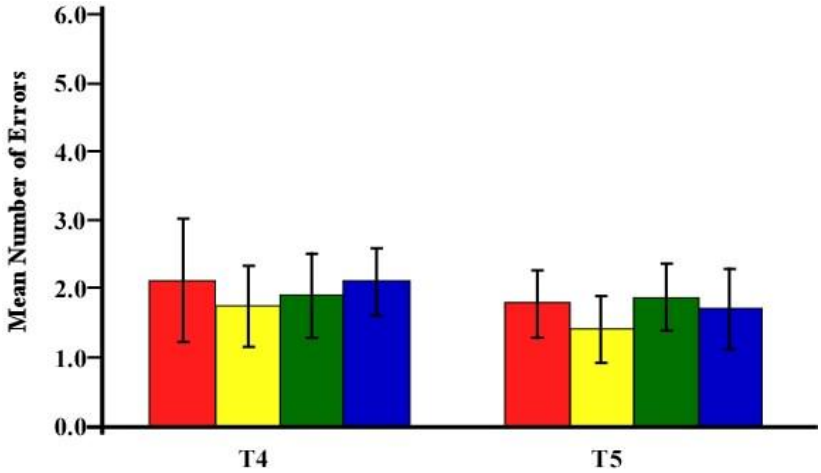

Figure 3: Pre-TBI RAWM errors for NT-Sham (red, n=9), NT-TBI (yellow, n=10), AD-Sham (green, n=9), and AD-TBI (blue, n=10) mice. T4 and T5 represent the average of the last two sessions for the fourth trial or fifth trial, respectively. Each bar denotes the group average of the last two days of Pre-TBI testing. Error bars represent standard error of the means.

Post-TBI RAWM Performance

After TBI surgeries, four AD-transgenic animals did not survive. Thus, the number of each group the surgery became as follows: AD-TBI (n = 7), AD-Sham (n = 8), NT-TBI (n = 10), and NT-Sham (n = 9).

Two Weeks After TBI

Figure 4 shows the mean numbers of errors for Post-TBI tests (A, 2 weeks; B, 4 weeks; C, 6 weeks after TBI).Two weeks after TBI exposure (Figure 4A), there was a main effect for T4 in terms of genotype, $F(1,33)=5.906$, $p=.021$, TBI exposure, $F(1,33)=4.927$, $p=.034$, and interaction of genotype by TBI exposure, $F(1,33)=7.167$, $p=.012$. AD-TBI mice made significantly more errors ($M=3.9$, $SD=1.90$) than all other groups: AD-Sham mice ($M=1.3$, $SD=1.49$, $t(13)=2.989$, $p=.01$); NT-TBI ($M=1.2$, $SD=1.11$, $t(15)=3.743$, $p=.002$); NT-Sham ($M=1.4$, $SD=1.70$, $t(14)=2.751$, $p=.016$).

For T5, there were no significant differences between AD and NT groups, $F(1,33)=.092$, $p=.763$, or between TBI and Sham groups, $F(1,33)=.108$, $p=.745$. The

interaction of genotype and TBI exposure for T5 was also not significant , $F(1,33)=.423$, $p=.521$.

Four Weeks After TBI

Four weeks after TBI exposure (Figure 4B), there were no significant differences for T4 between AD and NT groups, $F(1,33)=.137$, $p=.714$, or between TBI and Sham groups, $F(1,33)=.569$. $p=.456$. Although the interaction of genotype by TBI exposure was significant, $F(1,33)=5.694$, $p=.024$, subsequent t-tests failed to show that AD-TBI mice made more errors ($M=3.2$, $SD=1.75$) than any other groups: AD-Sham mice ($M=1.4$, $SD=1.60$, $t(13)=2.127$, $p=.053$); NT-TBI ($M=1.6$, $SD=1.60$, $t(15)=1.973$, $p=.067$); and NT-Sham ($M=2.6$, $SD=1.83$, $t(14)=0.728$, $p=.479$). For T5, there was no main effect for genotype, $F(1,33)=.133$, $p=.718$, TBI exposure, $F(1,33)=4.193$, $p=.049$, or interaction of genotype and TBI exposure, $F(1,33)=.014$, $p=.908$.

Six Weeks After TBI

Six weeks after TBI exposure (Figure 4C), there was a main effect for T4 in terms of genotype, $F(1,33)=4.441$, $p=.044$, TBI exposure, $F(1,33)=4.998$, $p=.033$, and interaction of genotype by TBI exposure, $F(1,33)=6.641$, $p=.015$. The AD-TBI mice made significantly more errors ($M=3.8$, $SD=1.52$) than all other groups: AD-Sham mice ($M=1.4$, $SD=1.52$, $t(13)=2.979$, $p=.011$); NT-TBI ($M=1.5$, $SD=1.31$, $t(15)=3.311$, $p=.005$); NT-Sham ($M=1.7$, $SD=1.32$, $t(14)=2.977$, $p=.010$).

For T5, there were no significant differences between AD and NT groups, $F(1,33)=.000$, $p=.986$. However, there were significant effects for TBI exposure, $F(1,33)=6.950$, $p=.013$, and the interaction of genotype and TBI exposure, $F(1,33)=5.269$, $p=.029$. On average, AD-TBI mice made significantly more errors

(*M*=2.6, *SD*=1.41) than AD-Sham mice (*M*=0.3, *SD*=0.37, *t*(13)=4.533, *p*=.001). However, the errors made by AD-TBI mice were not significantly different from the errors made by NT-TBI mice (*M*=1.6, *SD*=1.54), *t*(15)=1.493, *p*=.156, or NT-Sham mice (*M*=1.7, *SD*=1.32), *t*(14)=1.603, *p*=.131.

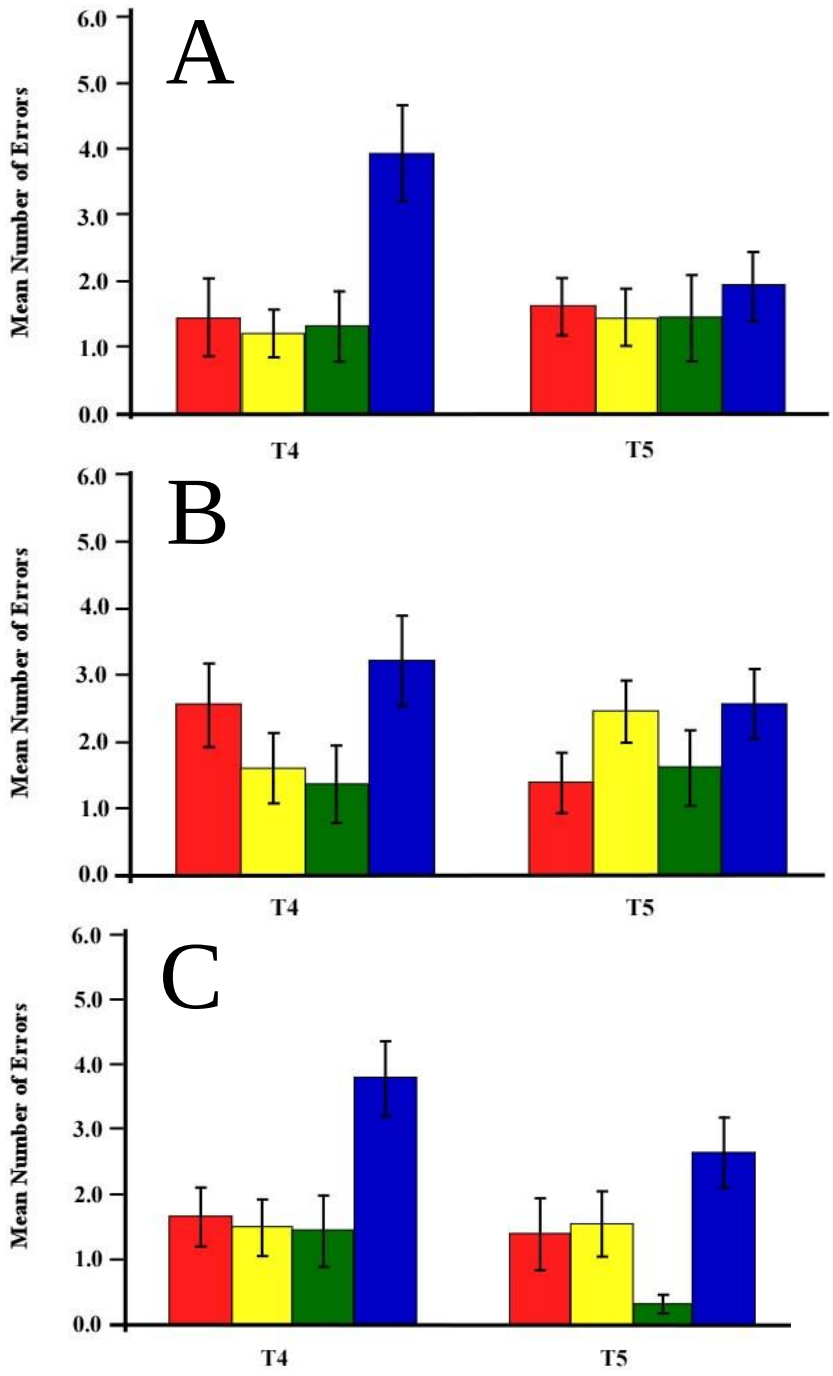

Figure 4: Post-TBI RAWM errors for NT-Sham (red, n=9), NT-TBI (yellow, n=10), AD-Sham (green, n=8), and AD-TBI (blue, n=7) mice. T4 and T5 represent the average of the last two sessions for the fourth trial or fifth trial, respectively. Each bar denotes the group average of the last two days of each Post-TBI testing (A, 2 weeks; B, 4 weeks; C, 6 weeks). Error bars represent standard error of the means.

Neurological Assessment

Figure 5 shows the results of the neurological assessment tasks (A, Balance Beam; B, String Agility; C, Visual Cliff). In balance beam task, there were no group differences for the amount of time before falling: genotype, $F(1,33)=.352$, $p=.557$, TBI exposure, $F(1,33)=2.240$, $p=.145$, or interaction of genotype and TBI exposure, $F(1,33)=.829$, $p=.370$. In the string agility task there was a significant difference in group performance, $\chi^2(3)=9.196$, $p=.027$. AD-Sham mice had zero successful attempts, whereas all other groups had successful and failed attempts at the task. There were no group differences for hesitation on the visual cliff task: genotype, $F(1,33)=1.430$, $p=.241$, TBI exposure, $F(1,33)=.001$, $p=.973$, or interaction of genotype and TBI exposure, $F(1,33)=2.644$, $p=.114$.

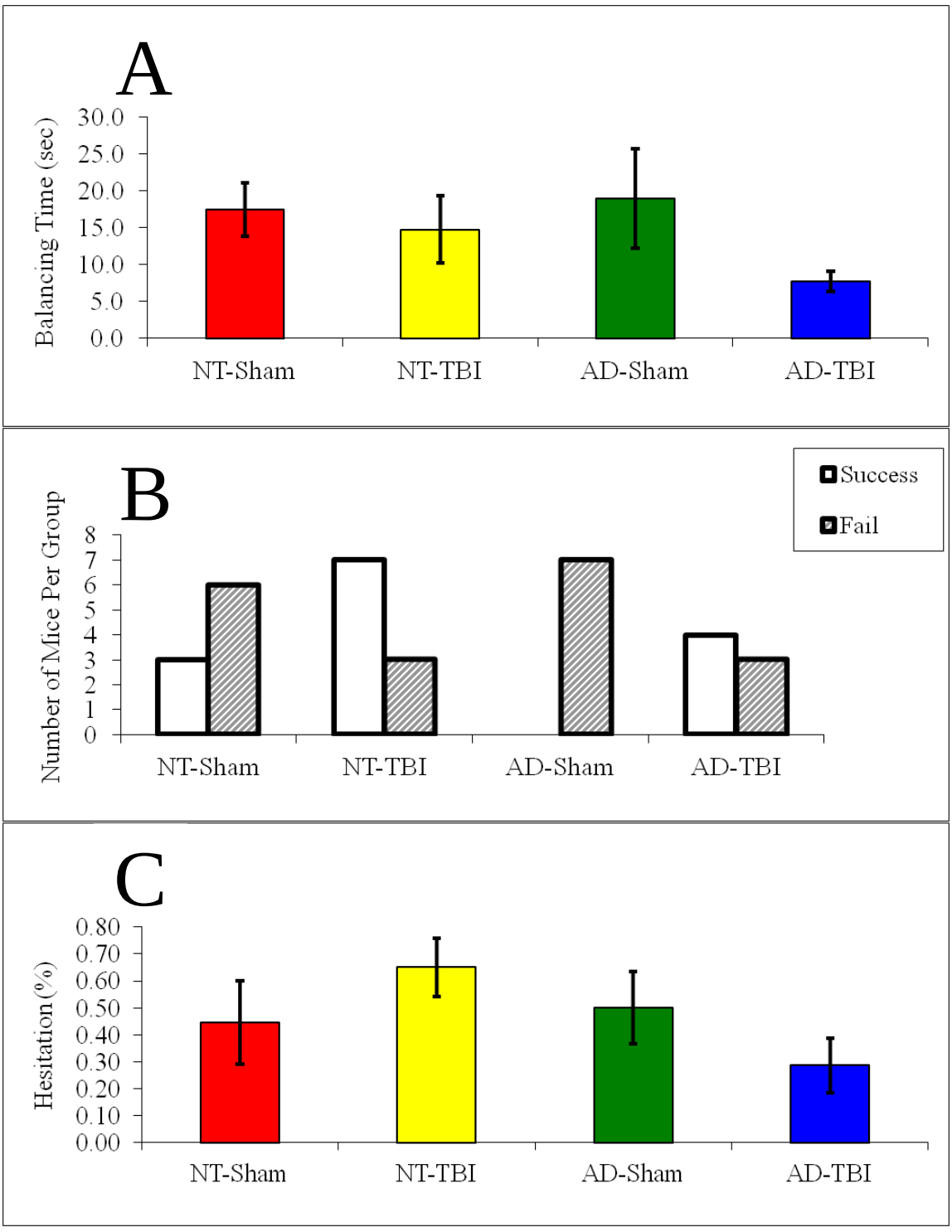

Figure 5: Neurological assessment tasks (A: Balance beam; B: String agility task; C: Visual cliff task). For A and C, bars represent the groups means of NT-Sham (red, n=9), NT-TBI (yellow, n=10), AD-Sham (green, n=8), and AD-TBI (blue, n=7) mice. Error bars represent standard error of the means. For B, bars represent the number of mice per group that were successful (solid white) or failed (stripes) to remain on the apparatus.

Experiment 2: Histological Examination

Lesion Reconstruction. After the experiment was completed, all mice were perfused and the brains were harvested. No brain damage was observed in either NT-Sham or AD-Sham groups. Mild to severe lesions were found in NT-TBI and AD-TBI groups. In Appendix A, Figures A1-10 represent the lesion reconstruction of NT-TBI mice; figures A11-17 represent the lesion reconstruction of AD-TBI mice.

Of the total of 10 NT-TBI mice, four NT-TBI mice had mild to moderate degrees of lesions in the dorsal cortex without significant damage in underlying structures including the hippocampus or subcortical areas. Animals belonging to this category are #12, #31, #37, and #59 (Figs. A2, A6, A7, and A10, respectively). Two NT-TBI mice had relatively severe lesions in the dorsal cortex as well as hippocampus, but without significant damage in other subcortical areas. Animals belonging to this category are #6 and #39 (Figs. A1 and A8). Four NT-TBI mice also had severe lesions in the dorsal cortex and hippocampus, but also in the subcortical areas. Animals belonging to this category are #13, #17, #28, and #42 (Figs. A3, A4, A5, and A9).

Of the total of seven AD-TBI mice, four AD-TBI mice had mild to moderate degrees of lesions in the dorsal cortex without significant damage in underlying structures. Animals belonging to this category are #20, #23, #27, and #38 (Figs. A14, A15, A16, and A17, respectively). Three AD-TBI mice had severe lesions including damage in the dorsal cortex, hippocampus, as well as the subcortical areas. Animals belonging to this category are #4, #11, and #16 (Figs. A11, A12, and A13).

Lesion Volume by Genotype. Figure 6 shows the lesion volume of cortical, hippocampal, and subcortical areas. There were no significant differences between lesion volume of AD- and NT-TBI mice, for cortex, $t(16)=0.992$, $p=.335$, corpus callosum, $t(16)=1.498$, $p=.153$, hippocampal area, $t(16)=1.591$, $p=.131$, subcortical area, $t(16)=1.958$, $p=.067$, or overall lesion volume, $t(16)=1.599$, $p=.129$.

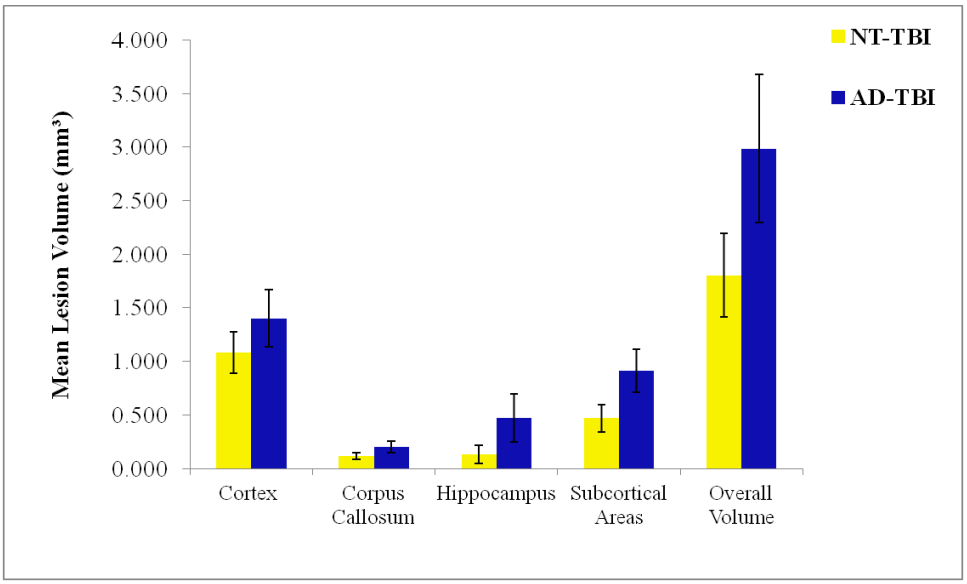

Figure 6: This figure represents the average lesion volume for four brain regions per group: AD-TBI (blue) and NT-TBI (yellow).

Immunohistochemistry. Antibody against Aβ 17-24 (4G8) showed reactivity in only one AD-TBI mouse (ID #4). No such reactivity was found in the rest of the AD-TBI mice, nor in the other conditions (AD-Sham, NT-TBI, and NT-Sham). The photographs in Figure 7 show no Aβ deposits in an AD-Sham mouse (A, #49) and Aβ deposits in the AD-TBI mouse throughout the cortex and hippocampus (B, #4). Aβ deposits are shown at a higher magnification (C). Figure 8 shows that the distribution pattern of the Aβ deposits in the brain using a camera lucida. All deposits were found bilaterally and widely in the cortex and hippocampus. No signals were detected in other subcortical regions, including the basal ganglia and thalamus.

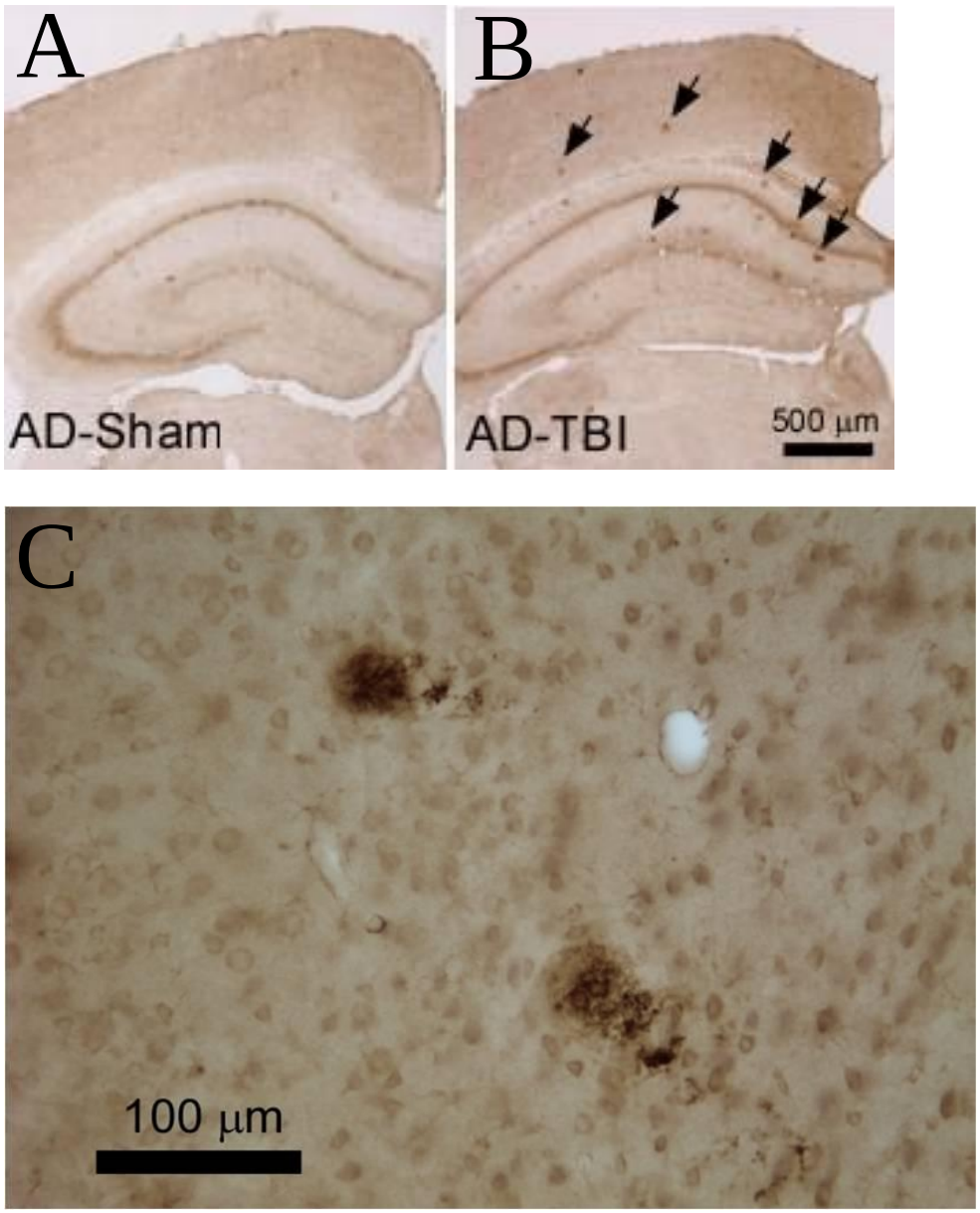

Figure 7: Photographs show no Aβ deposits in an AD-Sham mouse (A) and Aβ deposits in the AD-TBI mouse (B). Aβ deposits are shown at a higher magnification (C).

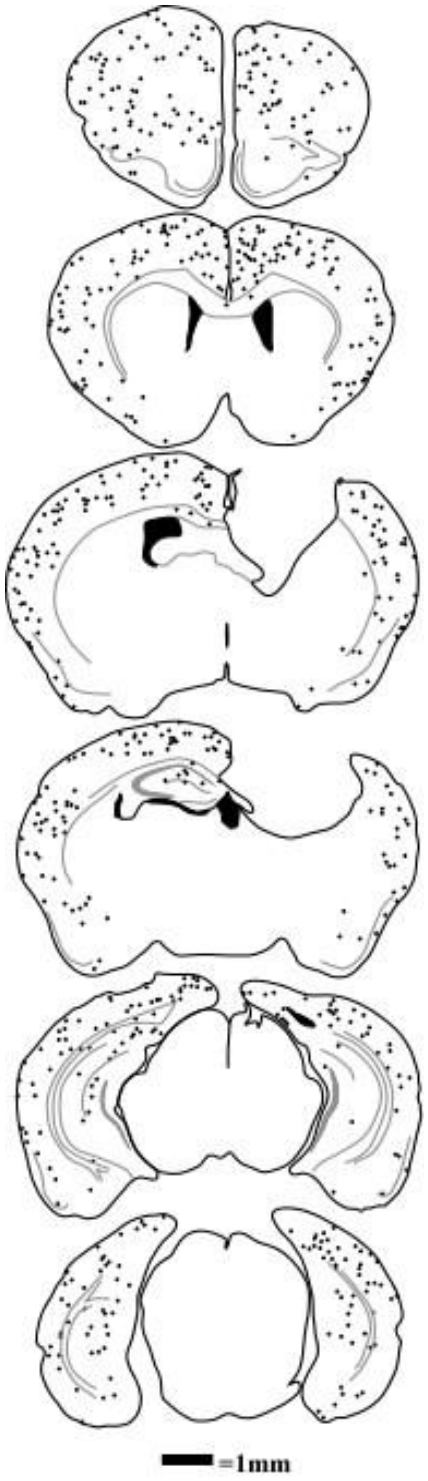

Figure 8: The distribution of Aβ deposits in MS 4 F10. The deposits were observed throughout the cortex and hippocampus.

Chapter 4:

Discussion

The results showed that AD-transgenic mice that received TBI showed more significant deteriorations in their behavioral performance in RAWM compared to AD-mice that received sham treatments as well as NT control mice. Thus, these findings support the hypothesis of Specific Aim 1 that TBI would affect cognitive/behavioral performance of AD-presymptomatic mice. This is the first study showing a significant behavioral effect of TBI on AD-transgenic animal models. Although previous studies did not show such effects on other AD-mice, I believe that the present results are reliable for the following reasons. First, there were no systematic behavioral differences in pre-TBI AD and NT groups, suggesting that AD-mice were not necessarily poor performers in the RAWM task compared to NT mice. Second, behavioral deficits of AD-TBI mice were already manifest two weeks after the TBI treatment, making more errors than all other groups. Although this effect was not clear at four weeks after TBI for some reasons, it became evident again at six weeks after TBI. Third, all subject animals were only five months old and AD-TBI mice still made more errors than other groups. Previous studies showed that such deficits in the tasks only appeared at 15-17 months of age (Arendash et al., 2001; Gordon et al. 2000). Finally, the neurological assessment showed that AD-TBI mice did not suffer impairments in balance, agility, or vision, despite their poor performance in the RAWM.

The results for Specific Aim 2 are not as clear as the behavioral findings. In terms of neuronal loss after TBI, AD-TBI and NT-TBI had similar lesion volumes and no statistically significant differences between the groups. In both groups, most neuronal damage was located in the dorsal cortex, as well as the hippocampus in some cases. However, it should be noted that lesion effects could be found not only in neuronal loss, but also in fiber damage, which was not measured in the present study. For instance, there were some individual cases (e.g., Figs. A11, A12, and A13), in which the hippocampus formation was shifted more posteriorly in the damaged hemisphere than in the intact hemisphere. It is possible that there was connection damage to these particular cases that our methods did not detect.

With respect to AD-like pathology, my prediction was that there would be more abundant and ubiquitous Aβ deposits in AD-TBI mice compared to AD-Sham, NT-TBI, or NT-Sham mice. However the results showed that only one AD-TBI mouse had AD-like pathology. There are two possible explanations for the lack of the expected results. First, it is possible that the results were indeed accurate and that AD-like pathology was not triggered by TBI in AD-transgenic (as well as all other) mice in the present study. If so, this means that the positive behavioral results were not directly associated with AD-like pathology. For instance, the AD-transgenic mice might have non-AD related cognitive problems that were augmented by TBI.

Second, the antibody and histological procedures used in the present study were somehow not sufficiently sensitive to detect the Aβ signals. Although the same type of antibody had been used for previous studies for AD-like histological analysis (biotin labeled Aβ 17-24 (4G8) monoclonal antibody), the antibody batch was different from

them and thus it is possible that this particular batch of antibody was not suited for the analysis. It is also possible that the analysis was hampered by other parameters of histological procedures, such as the perfusion, fixative solutions, duration of fixation, and dilution of antisera.

A recent study (Dr. Cesario Borlongan, personal communication) showed that the latter explanation is most likely. Thus, a similar histological analysis was conducted using the same AD-transgenic mice and the brain tissues were analyzed by using a different antibody (rabbit polyclonal anti-beta amyloid, 1:100, Abcam ab2539). The results showed that AD-TBI mice had higher numbers of Aβ positive cells in the hippocampus, compared to control groups (AD-Sham, NT-TBI, and NT-Sham). This group also used an antibody against MAP2 (1:500, Abcam, ab11267) to compare the differences in neurogenesis in these same mice. AD-TBI mice had decreased neurogenesis in the hippocampus compared to controls. Similar histopathological results were reported by Tran, LaFerla, Holtzman, & Brody (2011) who used 3x-transgenic mice. In sum, the present results showed that TBI precipitates AD in terms of behavior. Although the present study could not confirm the histopathological impact of TBI, these subsequent studies suggest that TBI indeed increased Aβ and possibly decreased neurogenesis in subject animals.

There are several other issues to be noted in the present study. First, animals with TBI continued to show behavioral deficits even after six weeks after the surgery, suggesting that the effect was not transitory, but sustained or even exacerbated through time. This observation may be important to understand the long-term effect of TBI in animals and patients. Future studies with prolonged testing periods may be valuable to

clarify this issue. Second, four mice died during the surgery and they were all AD-transgenic mice. Although the fatality of 4/19 compared to 0/19 of NT mice may not be statistically significant, it may be an indication that AD-transgenic mice were vulnerable to surgical procedures, such as anesthesia and bleeding. Finally, those AD-transgenic, as well as NT, mice that survived TBI all appeared healthy in a cursory glance, despite their deficit in a spatial learning task. Future studies should continue to determine whether the deficits are limited to the memory task, or other aspects of cognitive and affective abilities.

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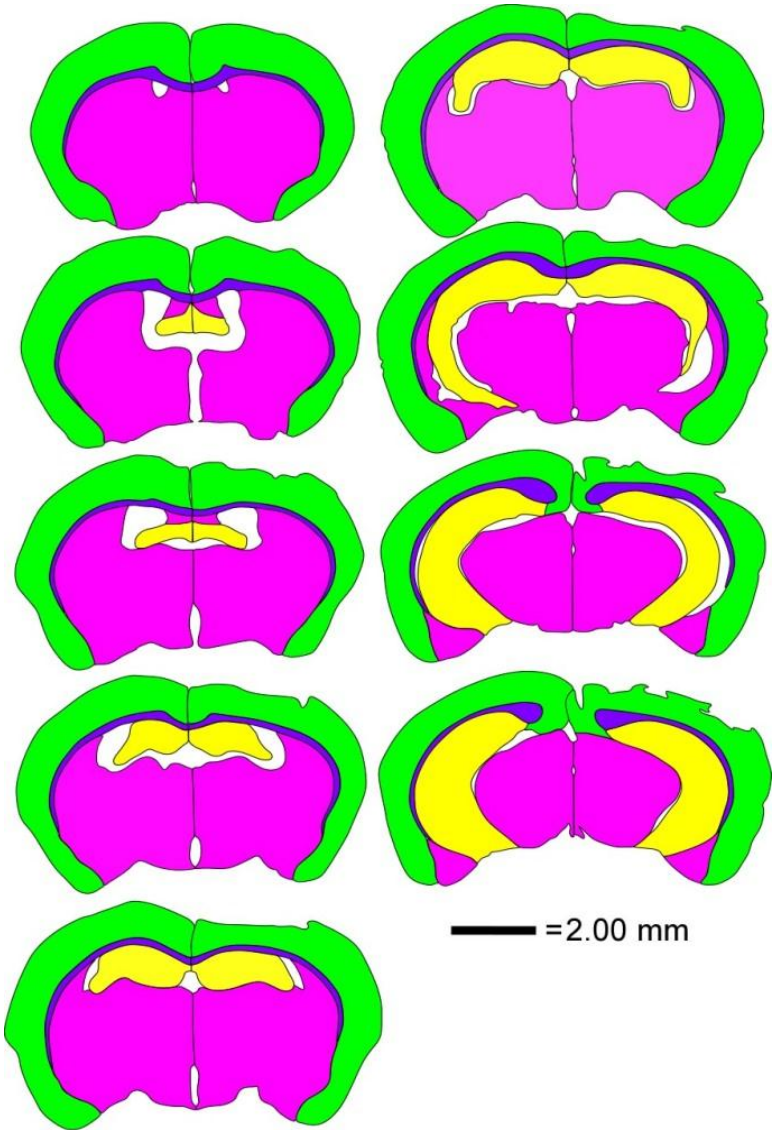
Appendices

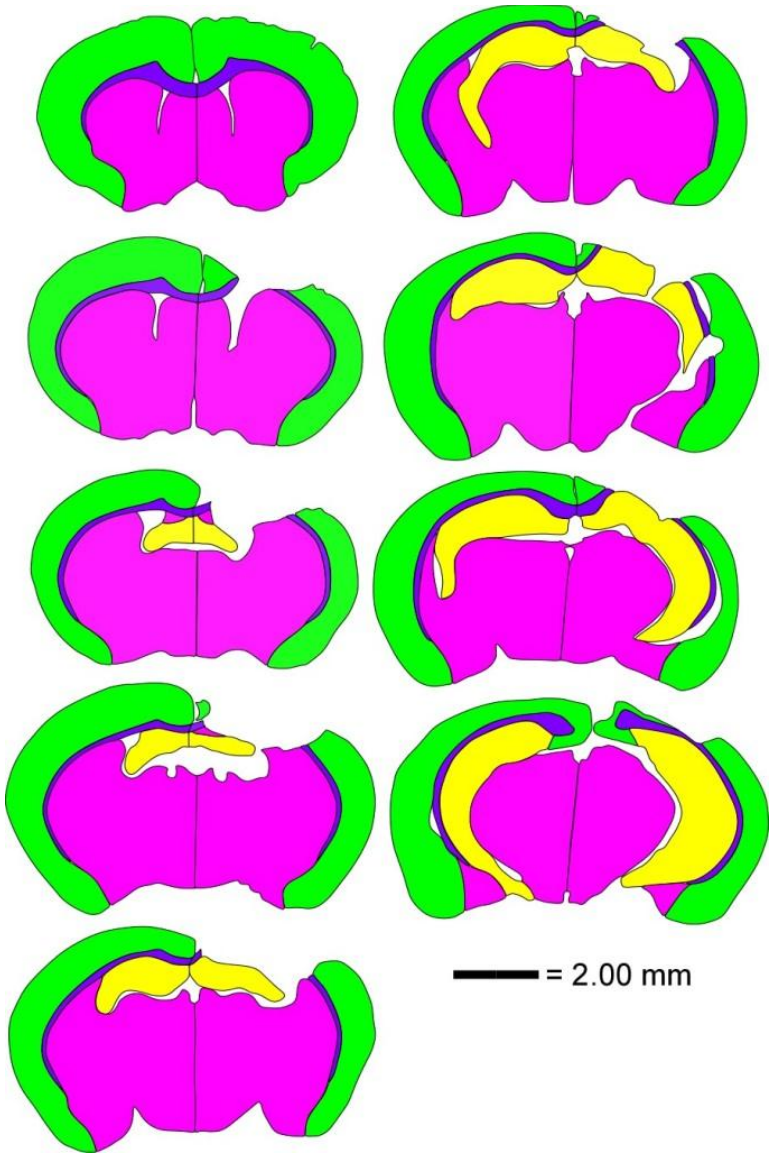
Appendix A: Extra Figures

Figure A1-A10 represent the lesion reconstruction for both NT and AD mice after TBI exposure. Figures A1-J1 represent NT-TBI Mice while figures K1-Q1 represent AD-TBI mice. Each figure is composed of transverse cut sections arranged from anterior to posterior. Each section is color coded to show the four brain regions of interest: cerebral cortex (green), corpus callosum (purple), hippocampus (yellow), and subcortical area (pink). Each scale bar = 2mm.



MS 6 NT-TBI

A1

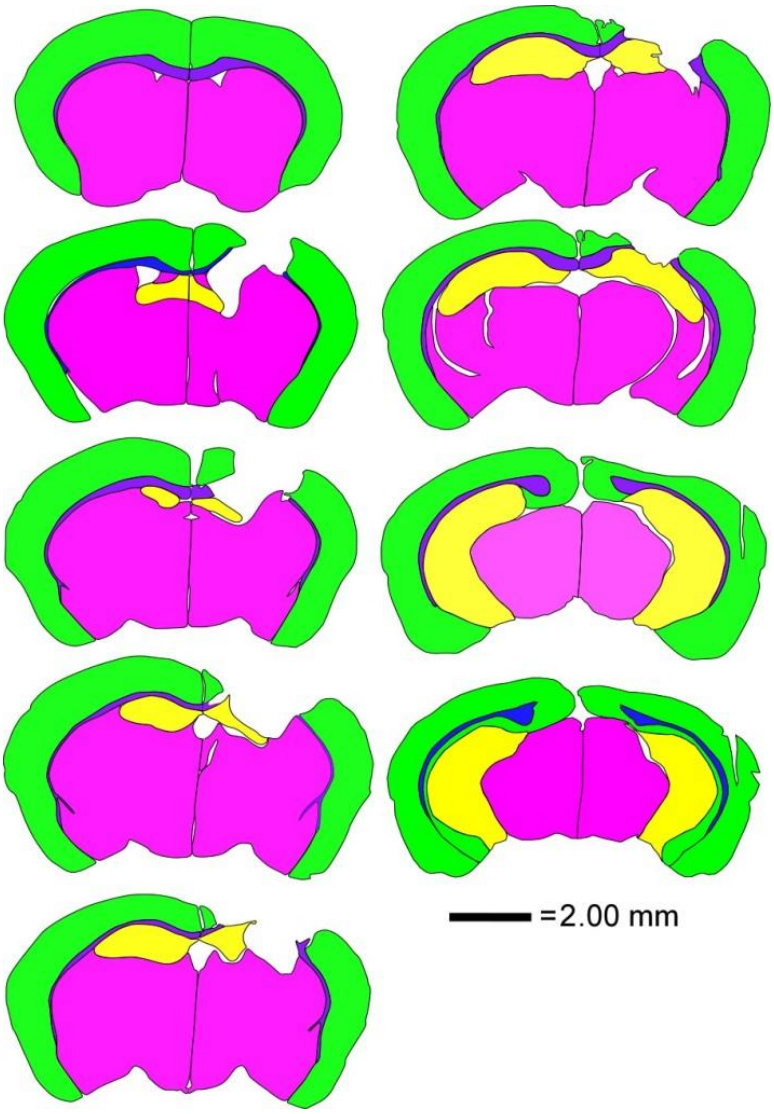


MS 12 NT-TBI

A2

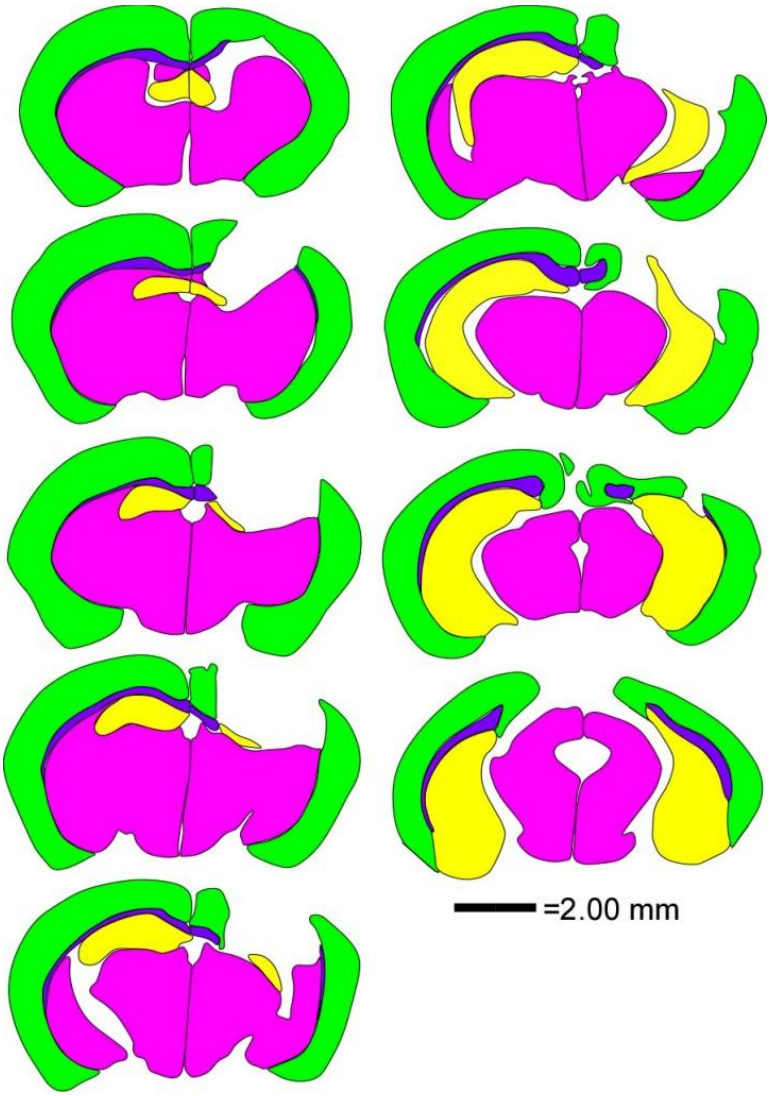
MS 13 NT-TBI

A3



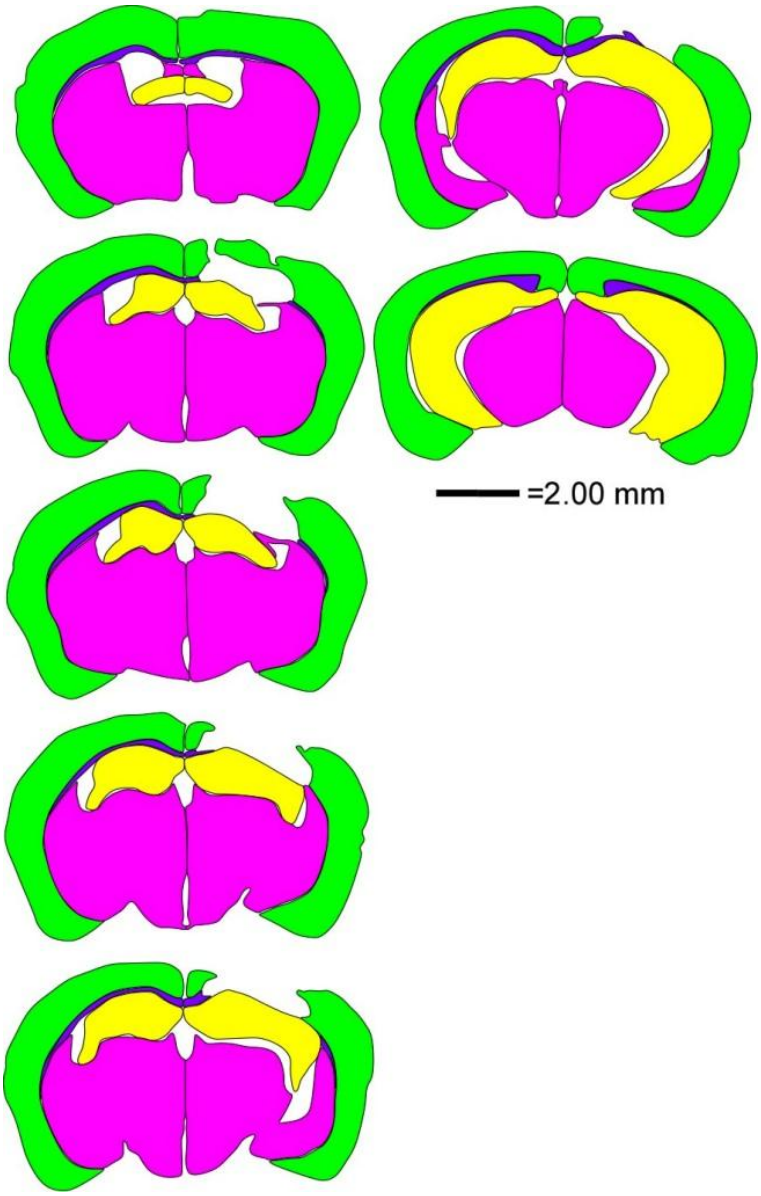
MS 17 NT-TBI

A4



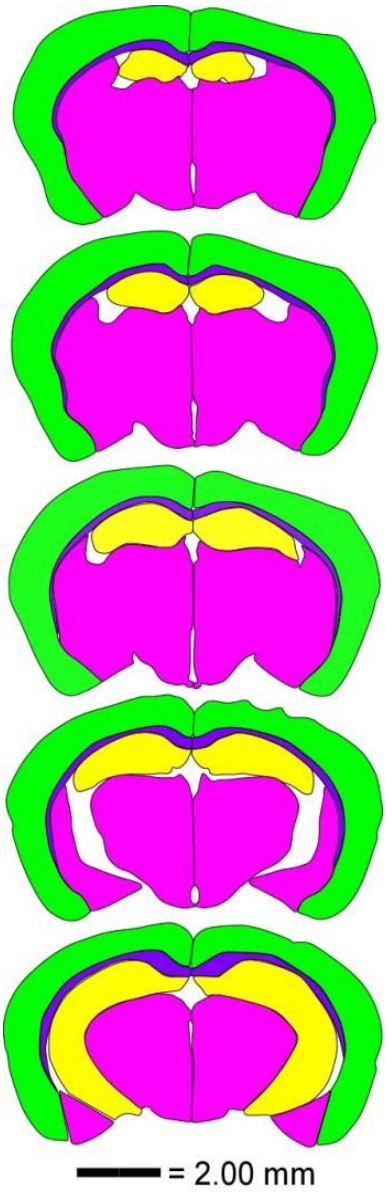
MS 28 NT-TBI

A5



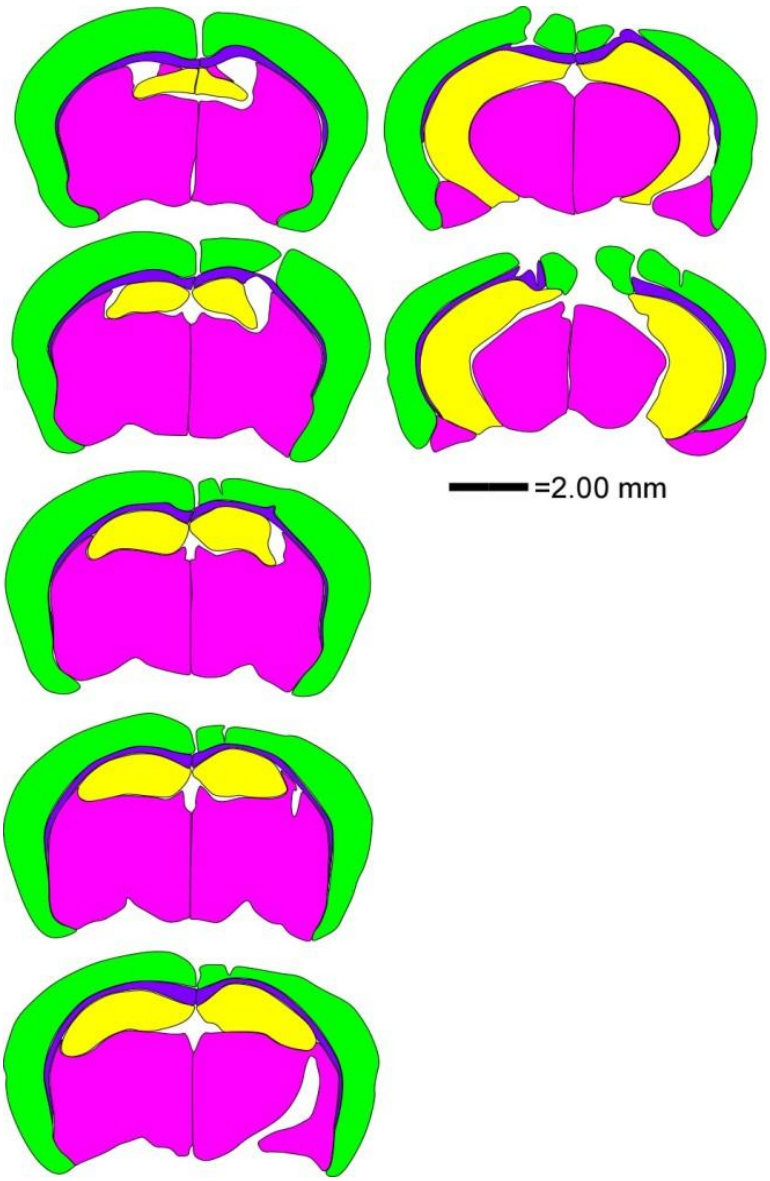
MS 31 NT-TBI

A6



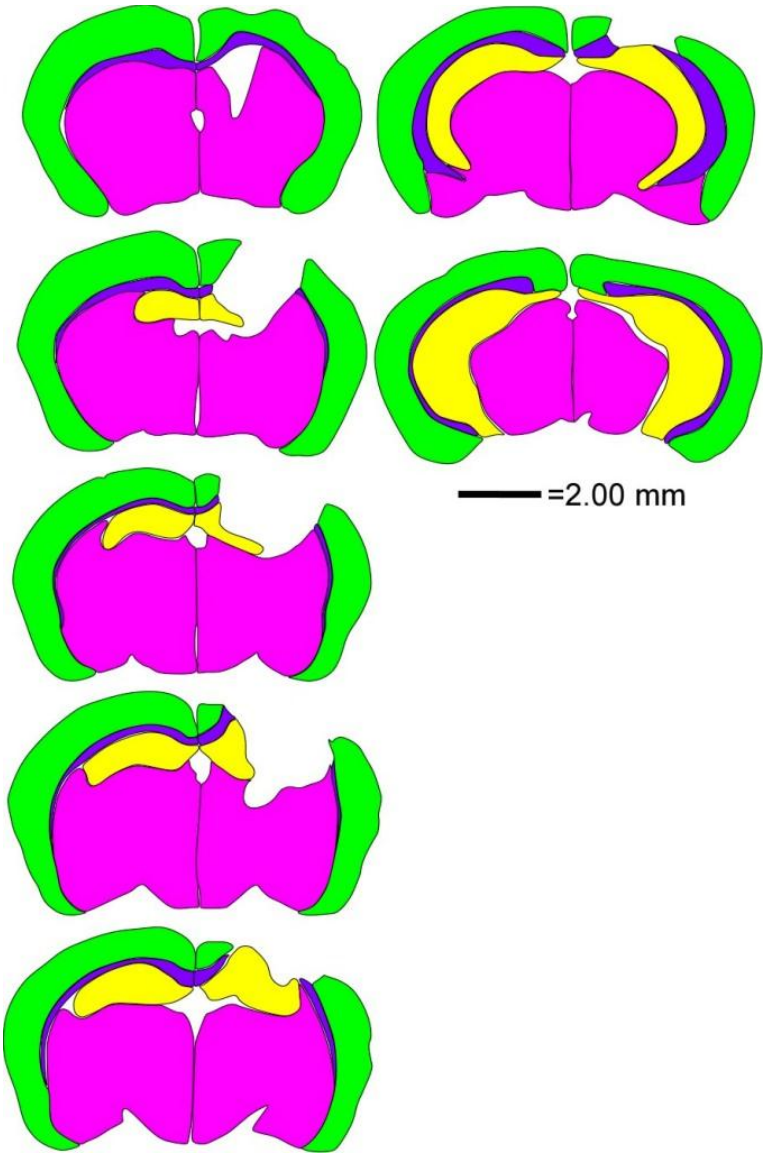
MS 37 NT-TBI

A7



MS 39 NT-TBI

A8

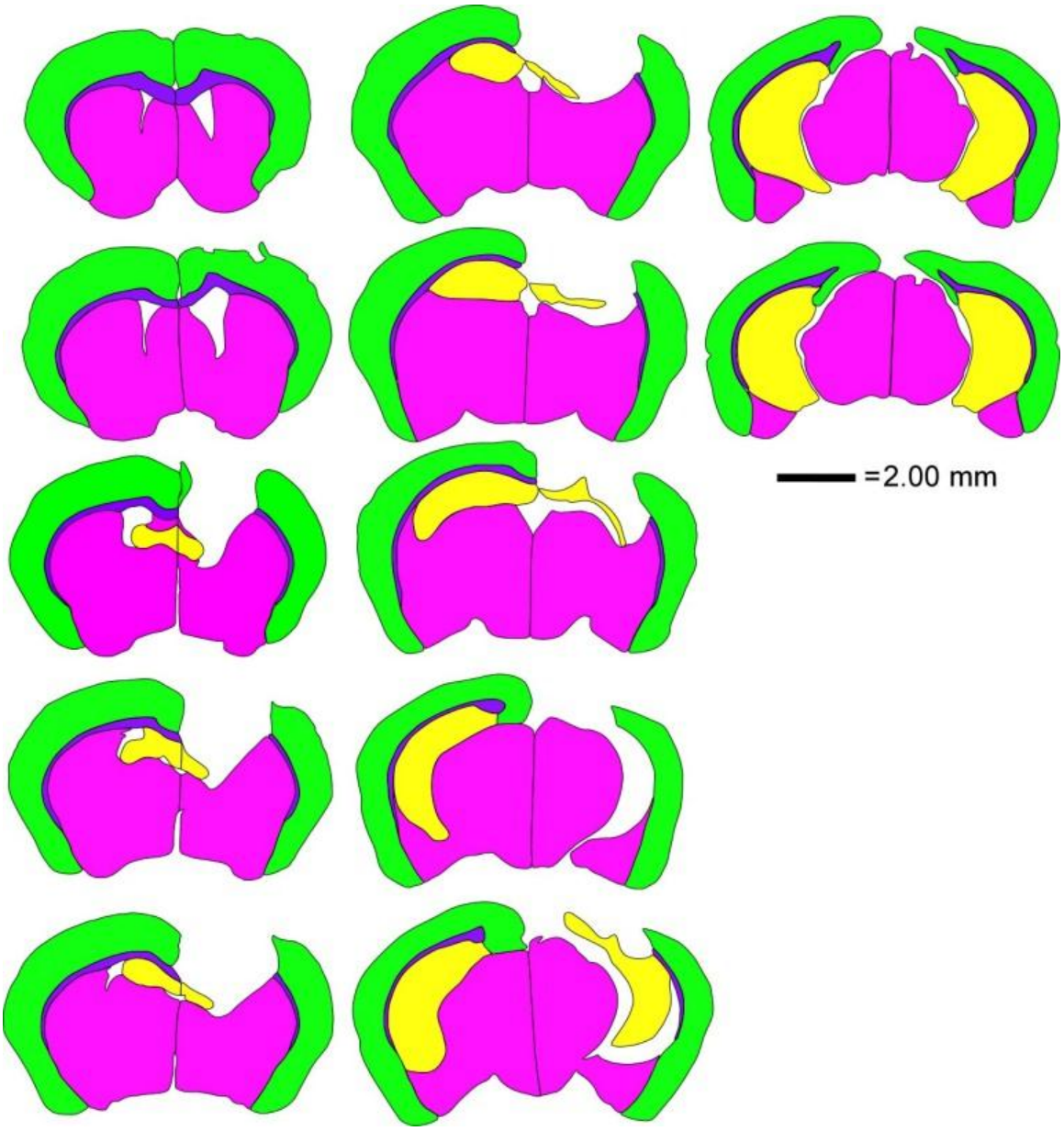


MS 42 NT-TBI

A9

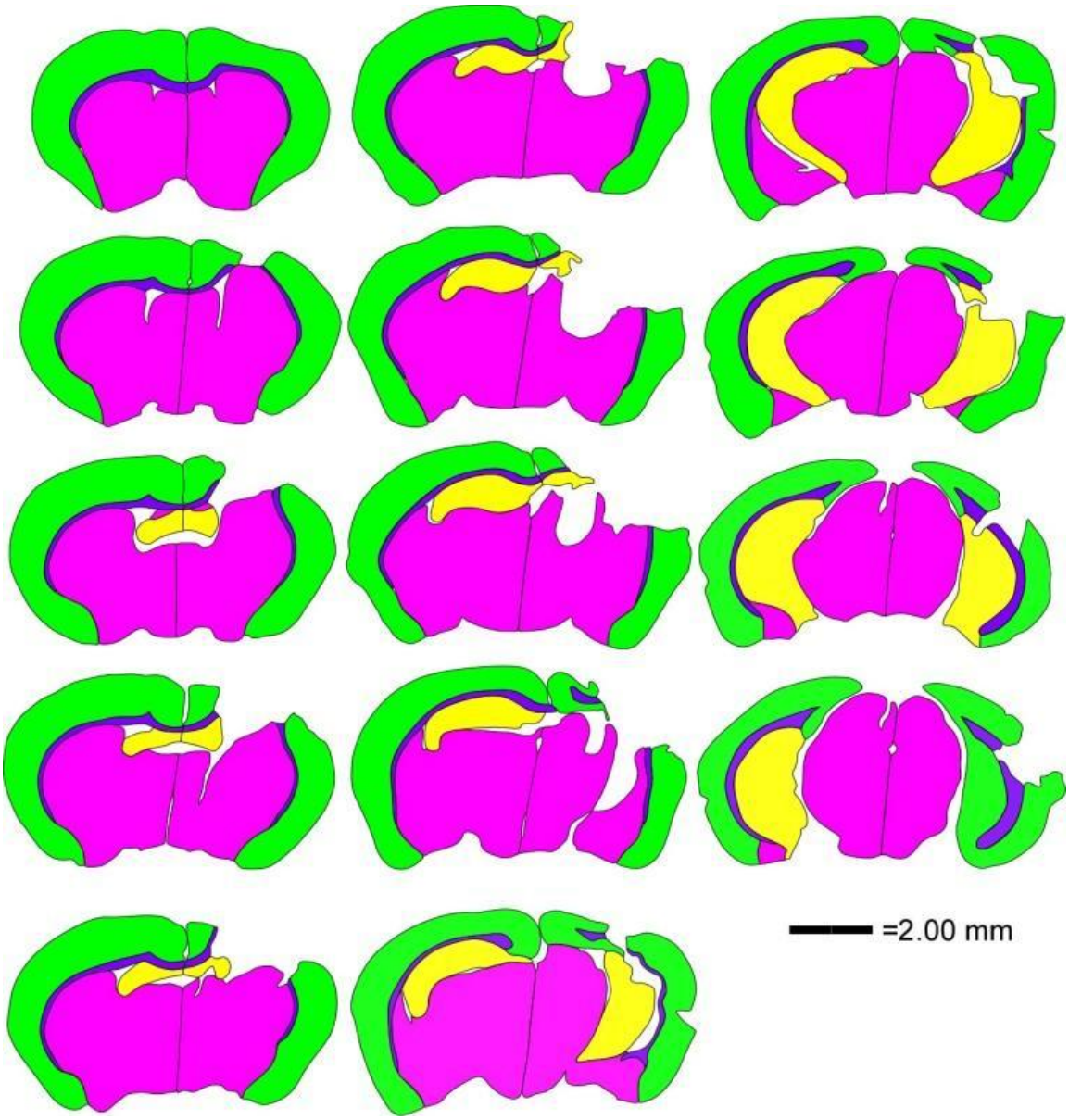
MS 59 NT-TBI

A10



MS 4 AD-TBI

A11

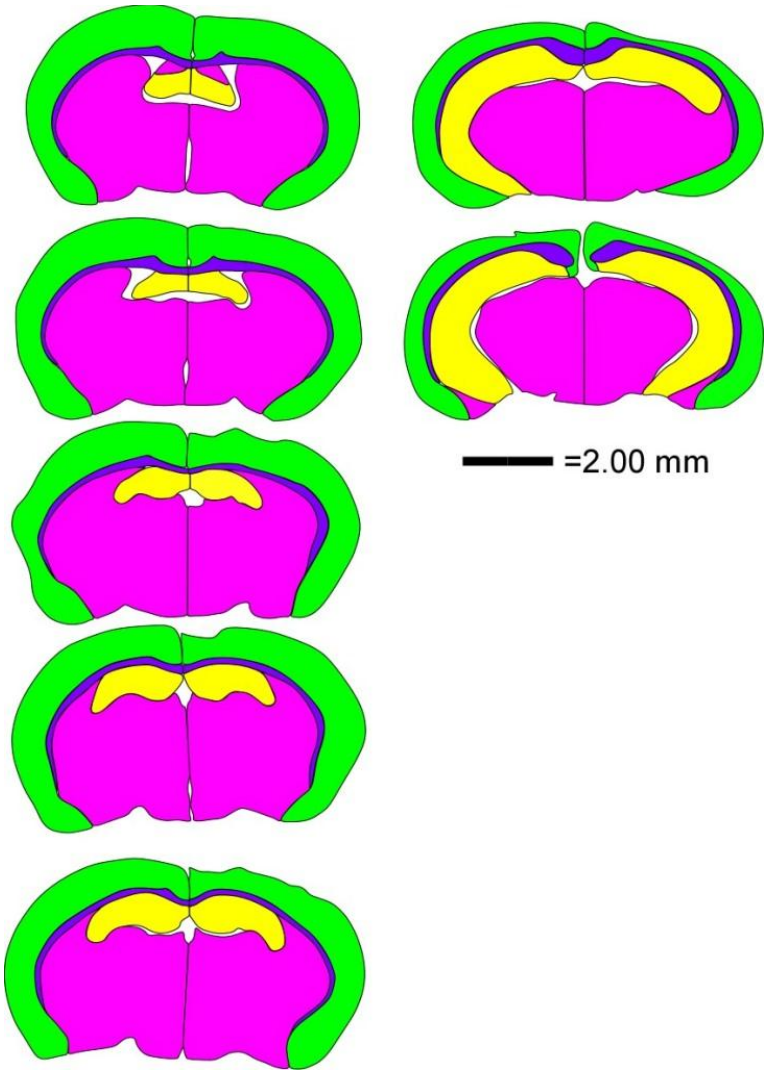


MS 11 AD-TBI

A12

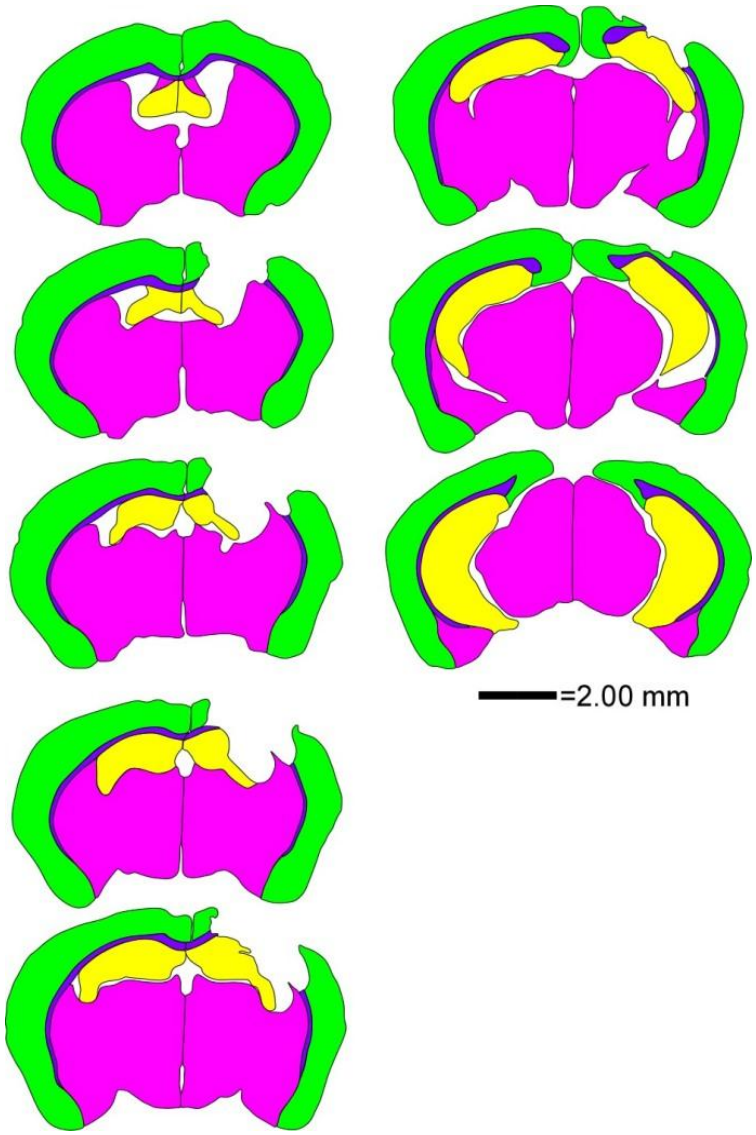
MS 16 AD-TBI

A13



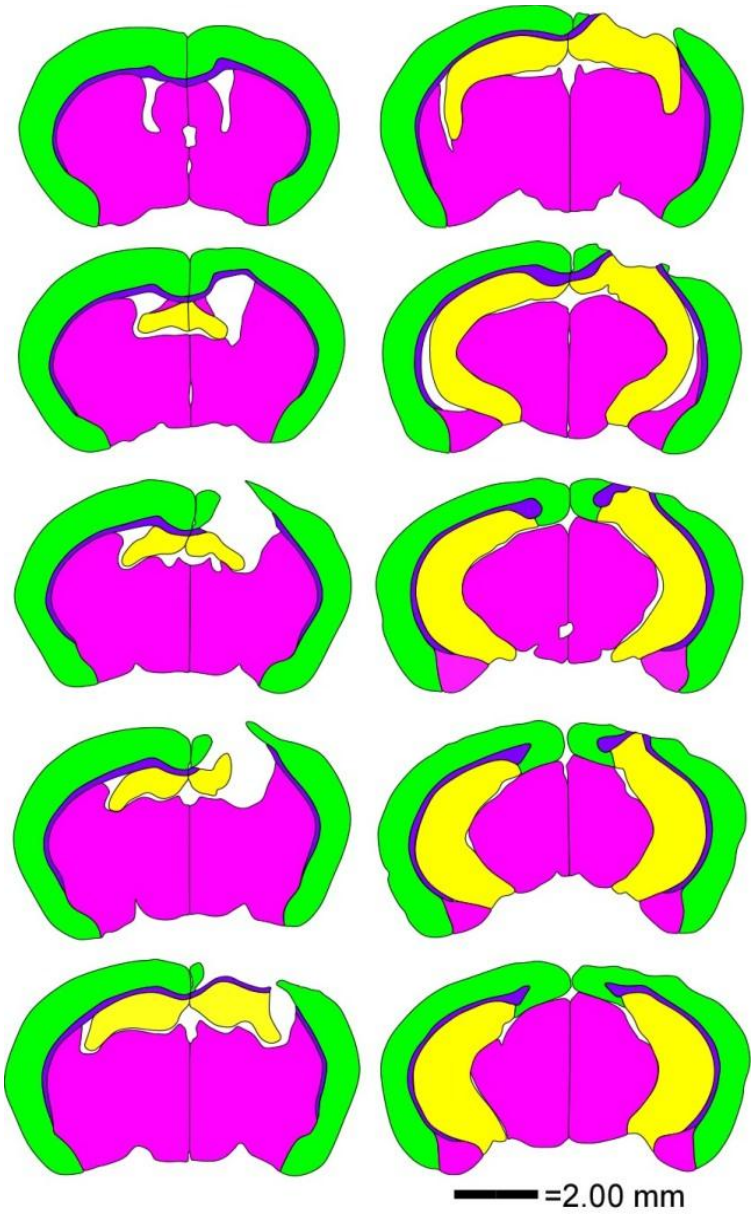
MS 20 AD-TBI

A14



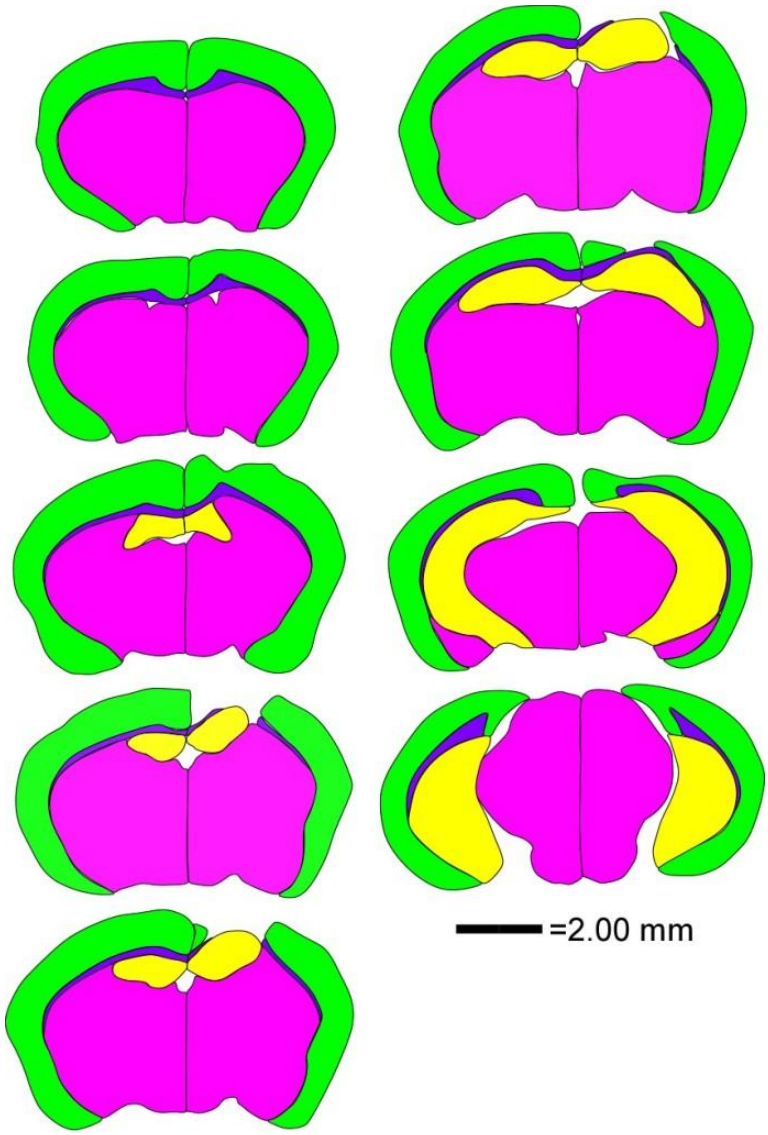
MS 23 AD-TBI

A15



MS 27 AD-TBI

A16



MS 38 AD-TBI

A17