RESEARCH ARTICLE

Delayed White Matter Injury in a Murine Model of Shaken Baby Syndrome

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Shaken baby syndrome, a rotational acceleration injury, is most common between 3 and 6 months of age and causes death in about 10 to 40% of cases and permanent neurological abnormalities in survivors. We developed a mouse model of shaken baby syndrome to investigate the pathophysiological mechanisms underlying the brain damage. Eight-day-old mouse pups were shaken for 15 seconds on a rotating shaker. Animals were sacrificed at different ages after shaking and brains were processed for histology. In 31-day-old pups, mortality was 27%, and 75% of survivors had focal brain lesions consisting of hemorrhagic or cystic lesions of the periventricular white matter, corpus callosum, and brainstem and cerebellar white matter. Hemorrhagic lesions were evident from postnatal day 13, and cysts developed gradually between days 15 and 31. All shaken animals, with or without focal lesions, had thinning of the hemispheric white matter, which was significant on day 31 but not earlier. Fragmented DNA labeling revealed a significant increase in cell death in the periventricular white matter, on days 9 and 13. White matter damage was reduced by pre-treatment with the NMDA receptor antagonist MK-801. This study showed that shaking immature mice produced white matter injury mimicking several aspects of human shaken baby syndrome and provided evidence that excess release of glutamate plays a role in the pathophysiology of the lesions.

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Introduction

Shaken baby syndrome, which can be associated with head impact, is most common between 3 and 6 months of age (2, 6) and is associated with a high mortality rate (10-40%) (22, 30), acute ophthalmologic and neurological signs, and poor neurological outcomes in more than half the cases (6, 13, 31, 36). Early appearing neurological problems include cerebral palsy and mental retardation, blindness, and epilepsy, while long-term follow-up studies have shown microcephaly associated with cognitive and major behavioral problems (5, 11, 28).

Parenchymal brain lesions associated with shaken baby syndrome include edema, bleeding, infarcts, white matter contusional tears, and axonal injury (6-9, 12, 22, 35, 37). The respective roles of acceleration-deceleration, head impact, edema and increased intracranial pressure in the genesis of these lesions have been the focus of considerable debate (1, 16, 29). Although head impact clearly has deleterious effects, shaking without head impact seems capable of producing brain lesions in infants (1, 13, 22, 31). On the other hand, the exact cellular and molecular links between shaking and the occurrence of brain lesions remain poorly understood. Animal models would be helpful in elucidating these links. Models of shaking-induced injury in adult primates and pigs have revealed diffuse axonal injury whose extent was directly proportional to the severity of shaking (14, 29, 32). Diffuse axonal injury was also produced in the adult rodent brain by contact loading or fluid-percussion insults (15, 24). However, the mechanical properties and plasticity of the developing brain differ markedly from those of the adult brain. Head shaking combined with severe hypoxia has been reported to produce brain lesions (33). However, we are not aware of models of head shaking without hypoxia during early postnatal development. Such a model would be useful for directly investigating the potential role of shaking, in the absence of detectable hypoxia, in the pathophysiology of brain lesions.

The goal of the present study was to establish, in developing mice, a model of isolated rotational shaken baby syndrome and to characterize the type, distribution, and course of the brain lesions. Furthermore, the study tried to distinguish between white matter and gray matter lesions. Finally, since excitotoxicity has been implicated in several developmental brain lesions including edema, stroke, hemorrhage, and impact-related head trauma (3, 19, 20), the potential pathophysiological role of excess glutamate release was investigated.

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Material and Methods

Animals and experimental design. Male and female Swiss mouse pups were used. The experimental protocols were approved by our institutional review board and met the guidelines of the INSERM. On postnatal day (P) 8, anesthetized pups were shaken during 15 seconds on a horizontally rotating shaker at a frequency of 900 cycles per minute. The pups were restrained by a device that neither caused chest compression nor limited head movements. Immediately after shaking, the pups were returned to their dams. No clinical evidence (changes in color skin, in breathing pattern, in alertness or in reactivity) of hypoxia-asphyxia was observed during or after shaking. The animals were divided into 3 groups: unshaken controls receiving a single intraperitoneal injection of phosphate buffer saline (PBS) on P8, pups shaken immediately after a single intraperitoneal injection of PBS, and pups shaken immediately after a single intraperitoneal injection of 1 mg/kg MK-801 (Tocris Cookson, Bristol, United Kingdom), a specific and potent inhibitor of NMDA receptors.

Histological procedures. The animals were decapitated on P9, P11, P13, P15, P19, or P31 (Table 1). The brains were removed immediately and fixed in 4% formalin for 7 days (for cresyl violet, Bodian and cell death staining, and for immunohistochemistry) or immediately frozen (for isolectin staining). Following paraffin embedding, serial 15-μm sagittal sections were cut throughout the brain. Every third section was stained with cresyl violet, and adjacent sections were used for immunohistochemistry or terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL). Cresyl violet-stained sections were analyzed for histological lesions by 2 investigators who worked independently from each other and were unaware of the groups to which the animals belonged.

Neocortical plate and underlying periventricular white matter thickness were measured quantitatively on P13, P19 and P31, using a blinded procedure. To avoid regional variations, the same anatomical level was examined in each group; this level was the sagittal section located 250 μm (on P13), 300 μm (on P19), or 500 μm (on P31) from the midline along the medial lateral axis. On these sections, images comprising the lateral ventricle and the neopallium were digitized using a CDD camera (Apogee Instruments Inc., Boston, Mass) to allow accurate measurement of cortical plate and white matter thickness along an axis perpendicular to and passing through the middle of the upper edge of the triangle-shaped lateral ventricle. In each group and at each study time-point, 10 to 12 animals (one section examined per animal) were included. Results were expressed as means±SEMs and were analyzed using the Student *t*-test or ANOVA with the Dunnett post-test.

Age of sacrifice	Controls	Shaken after PBS	Shaken after MK-801
P9	5	5	0
P11	10	15	0
P13	9	15	0
P15	4	8	0
P19	5	8	0
P31	48	56	15

Table 1. Numbers of brains subjected to histological examination.

In order to explore the presence of retinal hemorrhages, eyes from shaken and control animals were dissected out at P11 and P13 and fixed in 4% paraformaldehyde for three days. After having removed the anterior part of the eyes, retinas were incubated with diaminobenzidine to reveal the endogenous peroxidase of red cells. Whole retinas were mounted flat in glycerol. Twelve eyes were examined in each experimental group.

TUNEL staining. Cell death was detected using an in situ cell death detection kit as instructed by the manufacturer (Roche, Meylan, France). In brief, sections were deparaffinized, treated for 20 minutes at 37°C with 20 mg/ml proteinase K, and incubated for two minutes on ice with 0.1% Triton X-100. DNA strand breaks were identified by using terminal deoxynucleotidyl transferase for 60 minutes at 37°C to label free 3′-OH termini with fluorescein-labeled nucleotides. Incorporated nucleotides were detected using an anti-fluorescein antibody conjugated to alkaline phosphatase, with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate toluidonium salt as the substrates. TUNEL staining was performed on sagittal sections containing cerebellar peduncles and obtained from shaken and control pups killed on P9 and P13. Five animals were included in each group and, for each brain, 2 sections were examined, each by 2 independent observers, using a blinded procedure. On each section, labeled nuclei were counted throughout the periventricular white matter and neocortical plate of the entire hemisphere. Results were expressed as means±SEM and compared using the Student *t*-test.

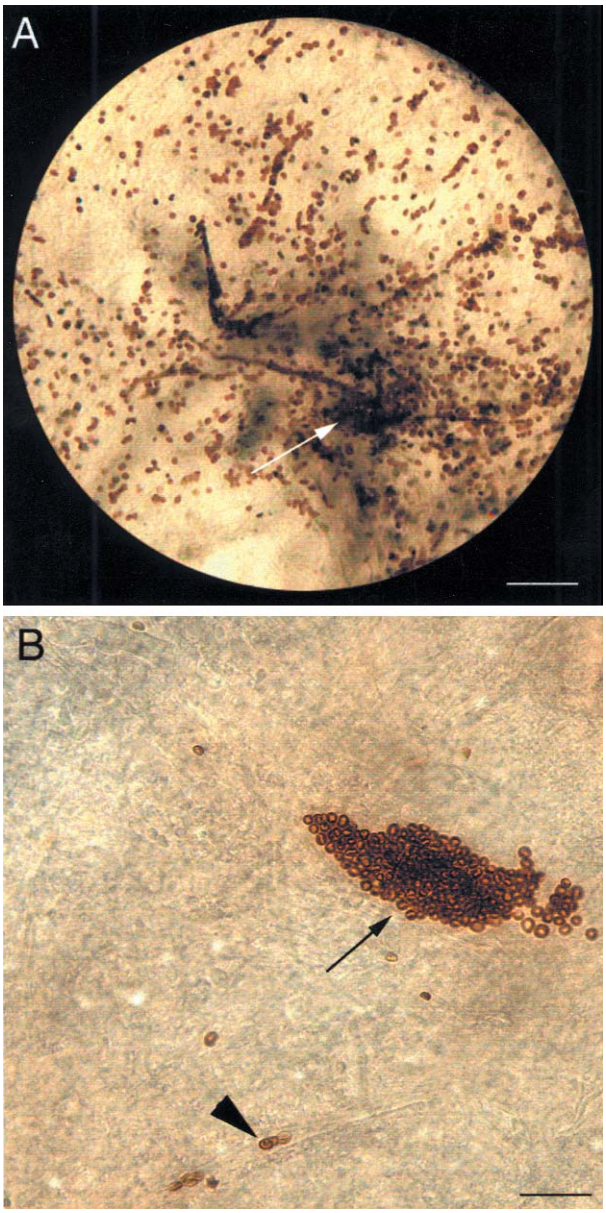

Figure 1. Shaking induced retinal hemorrhages. **A.** Low magnification of a whole mount retina from a P11 shaken animal showing labeled red cells in blood vessels and in a typical retinal hemorrhage (white arrow). **B.** Higher magnification of a retina from a P11 shaken pup showing another retinal hemorrhage (small black arrow) and a normal retinal blood vessel (black arrowhead). Bar = 150 μm (**A**) or 40 μm (**B**).

Immunohistochemistry and histochemistry. Deparaffinized brain sections from shaken and control animals killed on P11, P13 and P31 were used for immunohistochemistry and Bodian staining. Sections immediately adjacent to the one used for thickness

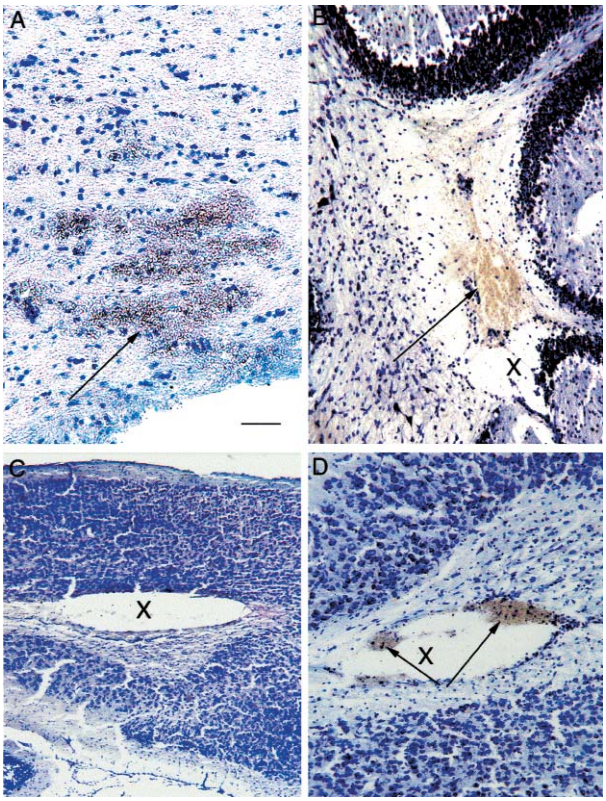

Figure 2. Focal white matter lesions induced by shaking on postnatal day 8 (P8). **A.** White matter hemorrhage (arrow) in the brainstem on P19. **B.** Cystic (X) and hemorrhagic (arrow) white matter lesion in the cerebellum on P19. **C.** Cystic white matter lesion (X) in the telencephalic hemisphere on P31. **D.** Cystic white matter lesion (X) with blood cells (arrows) in the telencephalic hemisphere on P31. Bar = 20 μm (**A**, **B** and **D**) or 40 μm (**C**).

measurements and without detectable histological lesions were stained with Bodian or immunoreacted overnight with polyclonal antibodies to glial fibrillary acid protein (GFAP) (Dako, Glosstrup, Denmark), myelin basic protein (MBP) (Roche), microtubule-associated protein type 2 (MAP-2) (Sigma, Saint Quentin Fallavier, France) or amyloid precursor protein (APP) (Sigma). These antibodies were detected using an avidin-biotin-horseradish peroxidase kit (Vector, Burlingame, CA), as instructed by the manufacturer. In each group, 10 pups (2 sections per brain) were studied qualitatively by 2 independent investigators working according to a blinded procedure.

Cryostat brain sections (adjacent to cresyl violet-stained sections without detectable histological lesions), 20 μm thick, from shaken and control animals killed on P11 and P13 were incubated overnight with biotinylated *Griffonea Simplicifolia* I isolectin B4 (Vector). Detection of labeled isolectin was performed with

avidin-biotin horseradish peroxidase kits (Vector), used as directed.

Results

Mortality, body weight and retinal hemorrhages. On P31, all control mice were alive ($n = 48$), whereas 54 (27%) of 200 shaken animals pre-treated with PBS died between P28 and P31, and one (6%) of the 16 shaken pups pre-treated with MK-801 died on P29. Most of the animals which spontaneously died were hypotrophic and lethargic at the time of death. In contrast, on P31, body weights of animals which did not spontaneously die were not significantly different between the 3 groups (controls, 21.0±0.7 g, $n = 48$; shaken animals pre-treated with PBS, 20.0±0.7 g, $n = 56$; shaken animals pre-treated with MK-801, 20.2±0.3 g, $n = 15$). When examined at P11 and P13, retinal hemorrhages were observed in about one third of eyes from shaken pups (Figure 1) while no hemorrhage was detected in control eyes.

Focal brain lesions. Histological analysis of serial brain sections from control animals did not reveal lesions at any of the ages studied (Table 1). Similarly, brains from shaken animals pre-treated with PBS killed on P9 or P11 showed no detectable lesions. In contrast, focal brain lesions (Figure 2) were found in large proportions of shaken animals killed on P13, P15, P19, or P31 (80% on P13, 75% on P15, 50% on P19, and 75% on P31 had one or more focal brain lesions). On P13, most animals displayed a single focal brain lesion, whereas at later time-points multifocal lesions were the rule. Focal white matter hemorrhages were the most common type of lesion on P13 and P15; focal white matter cysts were first noted on P15 and were the predominant lesion type from P19 onward. At each studied age, a single shaken brain had a destructive neocortical plate lesion.

A detailed analysis of the 42 PBS pre-treated shaken animals with focal brain lesions showed that the white matter lesions were located in the cerebellum (57% of brains), hemispheric white matter (31%, with involvement of the occipital lobes in 7 cases, parietal lobes in 4, and frontal lobes in 2), brainstem (24%), and other areas (1 %) including the olfactory bulbs, hippocampus, and diencephalon. Analysis of brains obtained from shaken animals which spontaneously died between P28 and P31 (see above) showed a pattern of lesion very similar to the pattern observed in shaken animals killed on P31, not permitting to detect a specific cerebral cause of death in these animals when compared to shaken animals which did not spontaneously die.

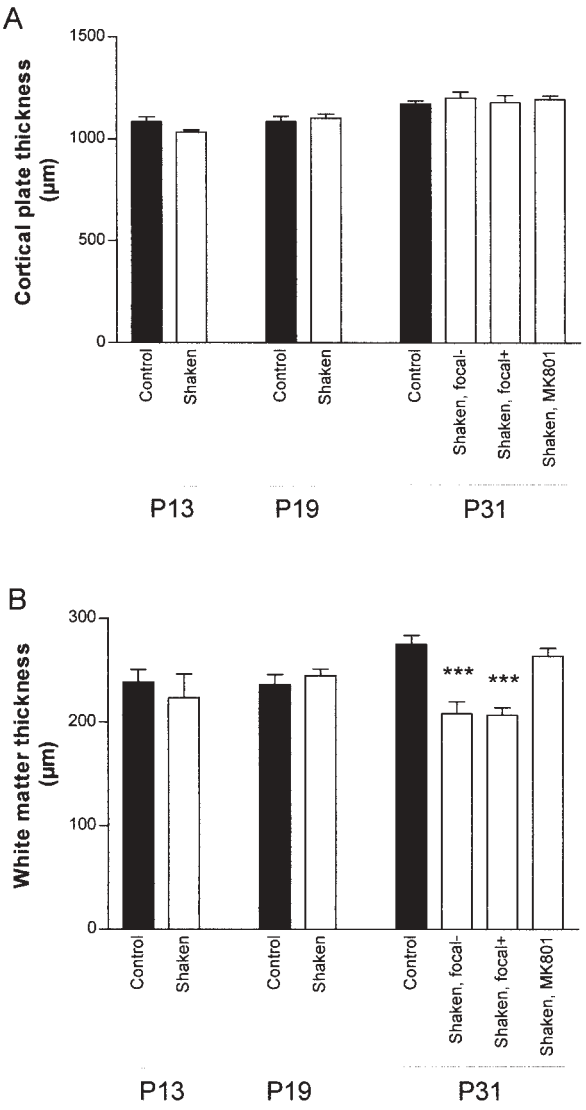

Figure 3. Shaking induced white matter thinning. Bars represent means of neocortical plate (**A**) and hemispheric white matter (**B**) thickness±SEM. Asterisks indicate statistically significant differences between controls and shaken animals (*** $p < 0.001$ in ANOVA with the Dunnett multiple comparison test). Controls, unshaken animals; Shaken, PBS pre-treated animals shaken on P8; focal +, PBS pre-treated shaken animals with focal white matter lesions on P31; focal -, PBS pre-treated shaken animals without detectable focal white matter lesions on P31; MK801, animals pre-treated with MK-801.

In MK-801 pre-treated animals, no white matter cysts were detected, although 6 animals had one (n = 4) or 2 (n = 2) pinpoint white matter hemorrhages in a limited area and one animal had a single, moderately severe hemorrhagic lesion in the periventricular white matter.

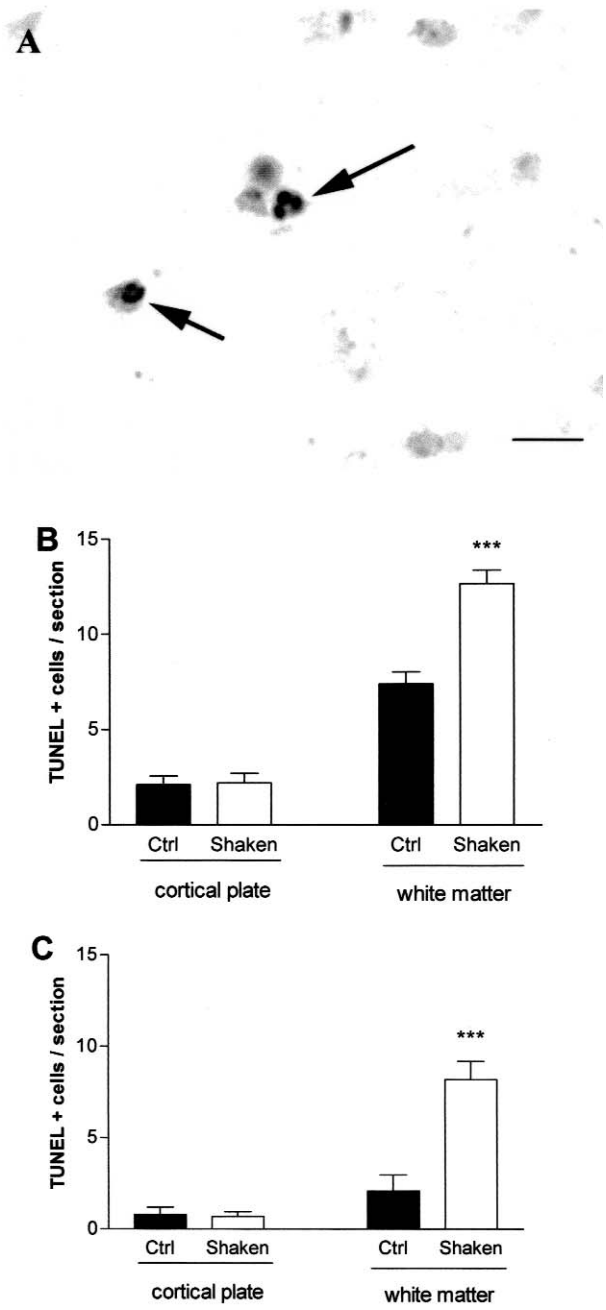

Figure 4. Shaking induced white matter cell death. **A.** TUNEL staining of brain sections from PBS pre-treated shaken animals killed on P13. Arrowheads point to examples of labeled nuclei. Bar=10 μm. B-C. Quantitative analysis of TUNEL-positive cell density on P9 (**B**) or P13 (**C**) in the neocortical layers and underlying white matter of control (closed bars) and PBS pre-treated shaken (open bars) animals. Bars represent mean values±SEM. Asterisks indicate statistically significant differences between controls and experimental animals (*** $p < 0.001$ in ANOVA with the Dunnett multiple comparison test).

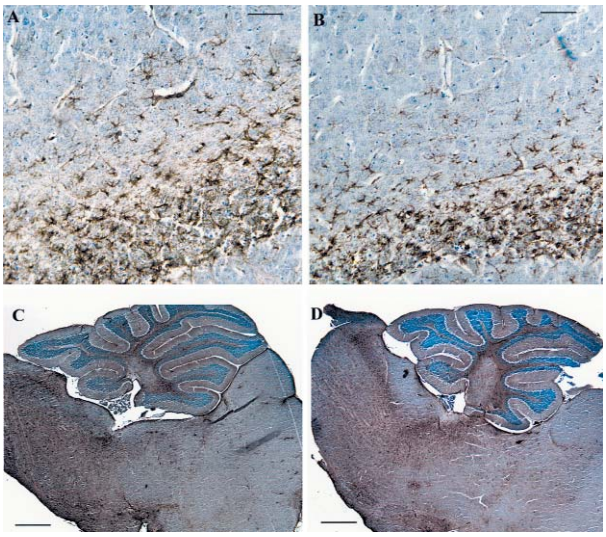

Figure 5. Shaking did not modify the density or distribution of astrocytes. Anti-GFAP immunostaining performed in P31 in control (**A**, **C**) and PBS pre-treated shaken (**B**, **D**) animals at the level of the neopallium (**A-B**) and, brainstem and cerebellum (**C-D**). Bar = 30 μm (**A-B**), 100 μm.

Neocortical plate and periventricular white matter thickness. At none of the studied ages (P13, P19 or P31) was neocortical plate thickness significantly different between the three groups (controls, PBS pre-treated shaken animals and shaken animals pre-treated with MK-801) (Figure 3A). In contrast, when compared to P31 controls, periventricular white matter thickness was significantly reduced in P31 PBS pre-treated shaken animals (Figure 3B); this white matter atrophy was of similar severity in PBS pre-treated shaken animals with and without detectable focal histological lesions (25% thickness loss in both groups). Periventricular white matter atrophy was not present in PBS pre-treated shaken animals killed at earlier time-points (P13 and P19) (Figure 3B). In animals pre-treated with MK-801, periventricular white matter thickness was not reduced as compared to the controls and was significantly greater than in the PBS pre-treated shaken animals (Figure 3B).

Neocortical plate and periventricular white matter cell death. When compared to control brains, brains from PBS pre-treated shaken mice showed a significantly increased amount of TUNEL-positive nuclei in the periventricular white matter on both P9 and P13 (Figure 4). In contrast, TUNEL staining of the neocortical plate at the same ages (P9 and P13) was similar in these 2 groups (Figure 4).

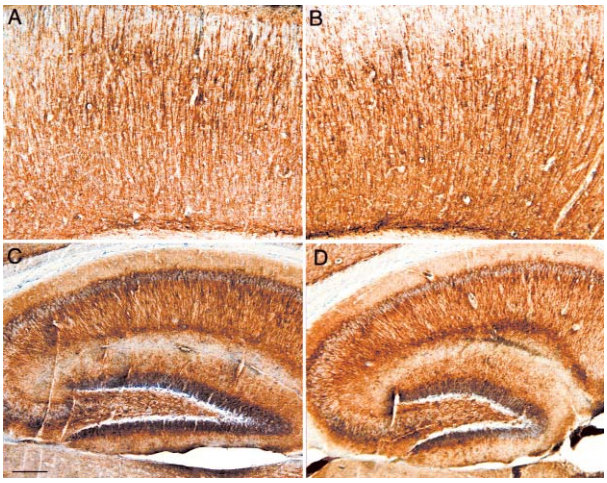

Figure 6. Shaking did not induce detectable differences in MAP-2 immunostaining. Sections from P31 controls (**A**, **C**) and shaken animals (**B**, **D**) at the level of the neocortex (A-B) and hippocampus (**C-D**). Bar = 20 μm.

GFAP, MAP-2, MBP, APP and Bodian staining. GFAP (an astroglial marker), MAP-2 (a marker of neuronal dendrites), MBP (a myelin marker), Bodian (an axonal marker) and APP (a marker of axonal degeneration) staining was performed on P11, P13, and P31 sections adjacent to cresyl violet-stained sections free of detectable focal lesions.

Qualitative analysis of brain sections containing identical anatomical structures and labeled with anti-GFAP (Figure 5), anti-MAP2 (Figure 6), anti-MBP (Figure 7A-B) antibodies or with Bodian (Figure 7C-D) showed no significant differences (distribution and intensity of labeling, morphology, distribution, organization and density of labeled structures) between controls and PBS pre-treated shaken animals. In contrast, APP immunostaining revealed a positive labeling of white matter tracts of shaken pups when compared to controls (Figure 8). This abnormal APP staining of white matter axons was already evident by P11, increased on P13 and was maximal on P31. White matter tracts in the neopallium, basal ganglia, cerebellum, and brainstem were affected in all studied shaken pups although the intensity of the immunolabeling was moderately variable between animals and, within a given brain, between white matter tracts.

Isolectin staining. *Griffonea Simplicifolia* I isolectin B4 (a marker of activated microglia-macrophages and endothelial cells) staining was performed on P11 and P13 sections containing identical anatomical structures and without detectable histological lesions. Qualitative analysis showed no significant difference of endothelial labeling between controls and PBS pre-treated shaken animals (data not shown). In addition, isolectin labeling

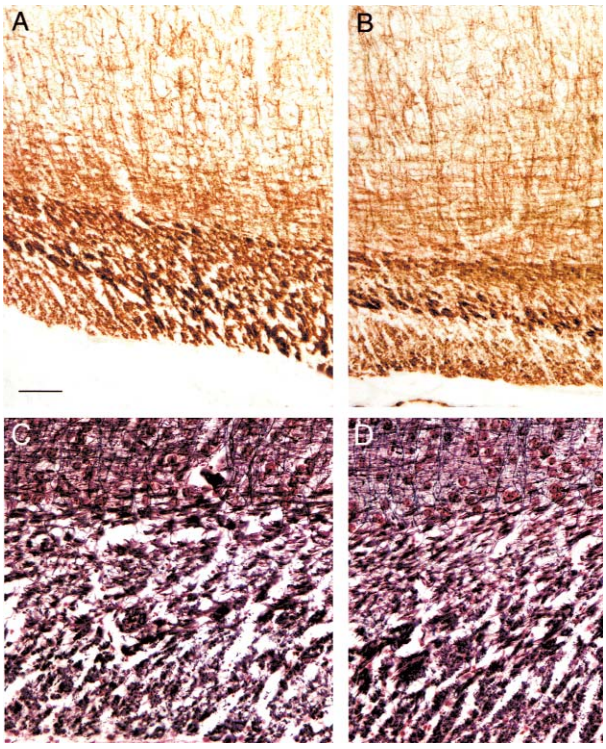

Figure 7. Density of white matter axons and myelin was not modified by shaking. MBP immunostaining (**A-B**) and Bodian staining (**C-D**) of P31 periventricular white matter from control (**A**, **C**) and shaken (**B**, **D**) animals. Bar = 40 μm (**A-B**) or 20 μm (**C-D**).

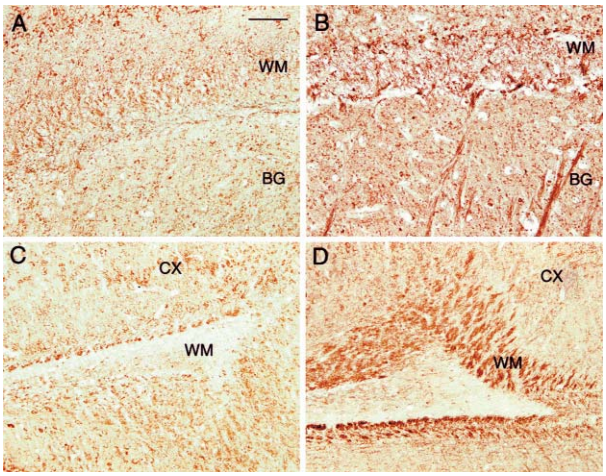

Figure 8. Shaking induced axonal damage. **A-B.** APP immunostaining of P11 parietal white matter from control (A) and shaken (**B**) animals. **C-D.** APP immunostaining of P31 occipital white matter from control (**C**) and shaken (**D**) animals. WM, white matter; BG, basal ganglia; CX, cortex. Bar = 40 μm.

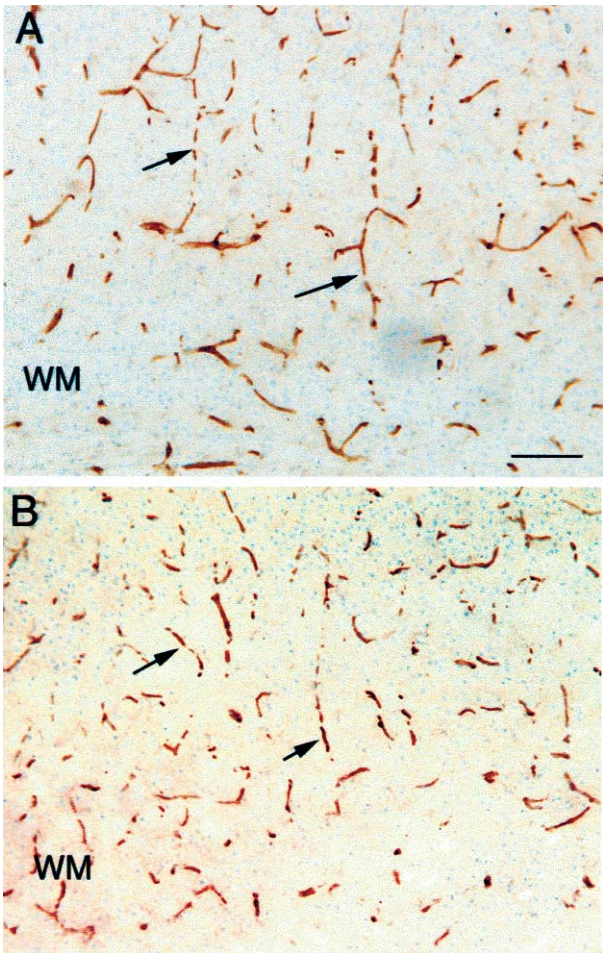

Figure 9. Shaking did not induce detectable *Griffonea Simplicifolia* I isolectin B4 labeling. Sections from P11 control (**A**) and shaken animal (**B**) at the level of the neopallium. Arrows point to examples of labeled blood vessels. WM, white matter. Bar = 40 μm.

did not reveal any activated microglial cells in white matter tracts or in the vicinity of blood vessels (Figure 9).

Discussion

The present study showed that, in developing mice, head shaking without impact and without detectable hypoxia induced mortality, multifocal white matter lesions in about two-thirds of survivors, and significant hemispheric white matter thinning. White matter damage was largely, but not completely, prevented by pretreatment with MK-801, a potent NMDA receptor antagonist.

The partial neuroprotective effects of MK-801 pretreatment suggest that NMDA receptor activation due to excessive release of glutamate played a role in the cascade that led to white matter lesions and thinning. In models of focal impact brain trauma in developing rat, several studies (3, 4, 19, 20) also found that NMDA receptor antagonists were neuroprotective. Similarly, several animal models including hypoxic-ischemic insults (18), excitotoxic insults (25, 34) or sustained seizures have established that the developing brain is remarkably sensitive to excess release of glutamate acting on NMDA receptors (for review, see 26).

Focal destructive white matter lesions, which were accompanied as expected by reactive astrogliosis, microgliosis, and abnormal MBP and APP staining (data not shown), probably contribute to the white matter thinning in shaken animals. However, the presence of a white matter thinning in shaken pups without detectable focal destructive white matter lesions suggest that other pathophysiological mechanisms are also participating to this white matter thinning. Axonal and myelin markers suggested that white matter thinning was associated with a harmonious reduction of the number of axons and amount of myelin. Although oligodendrocytes were not directly studied, the normal myelination of preserved axons in shaken animals argue for the survival of a sufficient number of oligodendrocytes to ensure this myelination. The APP immunostaining confirmed the axonal involvement in shaken pups. The lack of reactive gliosis and reactive microglia-macrophages in atrophic white matter tracts (at distance from focal destructive white matter lesions) argued against a clastic or destructive mechanism; they could be in favor of a phenomenon related to or sharing features with the physiological axonal pruning which normally occurs during postnatal brain development (for review, see 21) or with the pathological axono-dendritic and synaptic pruning observed in Rett syndrome (23), two developmental situations which do not induce any inflammation response of glial scar. Head shaking during the period of brain development could therefore lead to white matter injury through a combination of two distinct pathways, one being common with adult brain trauma (destructive lesions) and the other being specific to the developmental period (axonal pruning).

In our study, the early appearance of retinal hemorrhages contrasted with delayed white matter hemorrhages, suggesting separate pathophysiological pathways. Retinal hemorrhages were likely linked to traumatic contusions of blood vessels. In contrast, the mechanisms underlying delayed hemorrhages remained elusive: changes in morphology or density of endothelial cells or delayed perivascular reactive microgliosis were not found in the present material at stages preceding the appearance of white matter hemorrhages sug-

gesting that other factors such as modifications of endothelial cell permeability could participate to the delayed white matter hemorrhages. Molecular mechanisms such as neurotransmitters, inflammatory factors including cytokines, endogenous peptides, growth factors or ion changes which have been implicated in other models of brain trauma (for review, see 27) could also play a role in the present model. Further studies, including measures of brain vessel permeability, will be necessary to fully characterize the pathophysiological mechanisms of these delayed white matter hemorrhages.

Interestingly, in our model of head shaking without impact, brain damage was almost entirely confined to the white matter. Several findings suggest that the gray matter was not a key target in our model: *i)* only one shaken animal had a detectable histological lesion in the gray matter (neocortical plate); *ii)* neocortical thickness was unchanged after shaking; *iii)* TUNEL staining in PBS pre-treated shaken animals was increased in the hemispheric white matter but not in the neocortical plate; *iv)* GFAP immunostaining did not show reactive gray matter gliosis, which would have suggested neuronal damage; and *v)* immunostaining for MAP-2, a marker of neuronal differentiation, revealed similar patterns of labeling in PBS pre-treated shaken and control brains. Similarly to our model, acute subdural hematoma produced experimentally by blood infusion in infant piglets caused selective white matter damage (30). In contrast, focal impact brain trauma in newborn rats induced extensive neuronal cell death in several gray matter structures but no evidence of primary white matter involvement (3, 4). In addition, predominant gray matter lesions have been reported in a rat model of neonatal head shaking (33). However, this discrepancy may be ascribable to 2 major differences between their model and ours: *i)* in that published study, the rat pups were shaken on three consecutive days as compared to a single day in our study; and *ii)* the rat pups were subjected to hypoxia during head shaking, whereas our pups were not subjected to hypoxia and showed no clinical evidence of hypoxia during the experimental procedure, although no measurent of oxygen level was performed in shaken pups. In human shaken baby syndrome, neuropathological and radiological studies have found variable associations of gray matter and white matter damage (8-10, 12, 17, 22, 35), although most of these studies did not clearly separate cases with or without associated impact. When extrapolating animal data to humans, an important consideration is differences between species, including brain weight, brain mechanical properties, relative white matter thickness, and muscle tone of the back of the neck.

A striking finding from our study is that the white matter lesions and thinning associated with shaking became detectable only after some days. The delayed appearance of white matter lesions in the present model fits well with the symptom-free interval that precedes the emergence of radiological and neurological signs in some human infants with shaken baby syndrome (5, 10). In aggregate, these data support the existence of a window for therapeutic intervention in this syndrome.

Although the present model recapitulates several aspects of human shaken baby syndrome, it is not, as any animal paradigm of a human disease, a full mimic of the human counterpart. In particular, the present study did not permit to assessment of the presence or absence of subdural/subarachnoid hemorrhages which are common features in the human shaken baby syndrome, although retinal hemorrhages, another common feature, were observed in the present model. On the other hand, human infants who die following severe shaking do not exhibit a symptom-free interval. Human shaken baby syndrome appears to be a complex and multifactorial disorder which will require the combination, rather than the opposition, of different and separate animal models in order to get a full picture of the pathophysiology of this devastating entity.

Acknowledgments

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