UNIVERSITE DE LAUSANNE - FACULTE DE BIOLOGIE ET DE MEDECINE

Brain Mind Institute, EPFL
Neurologische Klinik, UniversitätsSpital Zürich

Sleep deprivation (SD) on focal brain ischemia in the rat: effects of different SD protocols

THESE

préparée sous la direction du Professeur Pierre Magistretti
(avec la co-direction du Professeur Claudio Bassetti)

et présentée à la Faculté de biologie et de médecine de
l'Université de Lausanne pour l'obtention du grade de

DOCTEUR EN MEDECINE

par

Hélène Julia JAEGER

Médecin diplômé de la Confédération Suisse
Originaire de Auboranges (Fribourg)

Lausanne

2013

UNIL | Université de Lausanne
Faculté de biologie
et de médecine

Ecole Doctorale
Doctorat en médecine

Imprimatur

Vu le rapport présenté par le jury d'examen, composé de

Directeur de thèse	*Monsieur le Professeur* **Pierre Magistretti**
Co-Directeur de thèse	*Monsieur le Professeur* **Claudio Bassetti**
Expert	*Monsieur le Docteur* **Andrea Rossetti**
Directrice de l'Ecole doctorale	*Madame le Professeur* **Stephanie Clarke**

la Commission MD de l'Ecole doctorale autorise l'impression de la thèse de

Madame Hélène Jaeger

intitulée

Sleep deprivation (SD) on focal brain ischemia in the rat: effects of different SD protocols

Lausanne, le 18 février 2014

pour Le Doyen
de la Faculté de Biologie et de Médecine

Madame le Professeur Stephanie Clarke
Directrice de l'Ecole doctorale

TABLE OF CONTENTS

ACKNOWLEDGMENTS

My warm thanks to Prof. Pierre Magistretti and Prof. Claudio Bassetti for the time they generously took to lead my MD thesis. I wish to add a special word of gratitude for Prof. Claudio Bassetti who gave me the opportunity to carry out this experimental work in his laboratory.

Many thanks also to Dr. Bo Gao who introduced me to preclinical research: her help was invaluable not only for the practical work, but also during the elaboration of this manuscript.

Finally, I would like to thank Dr. Ertugrul Cam: his technical support for surgery was immensely appreciated.

ABSTRACT

Sleep-wake disturbances are frequently observed in stroke patients and are associated with poorer functional outcome. Until now the effects of sleep on stroke evolution are unknown. The purpose of the present study was to evaluate the effects of three sleep deprivation (SD) protocols on brain damages after focal cerebral ischemia in a rat model.

Permanent occlusion of distal branches of the middle cerebral artery was induced in adult rats. The animals were then subjected to 6h SD, 12h SD or sleep disturbances (SDis) in which 3 x 12h sleep deprivation were performed by gentle handling. Infarct size and brain swelling were assessed by Cresyl violet staining, and the number of damaged cells was measured by *t*erminal deoxynucleotidyl *t*ransferase mediated d*U*TP *n*ick *e*nd *l*abeling (TUNEL) staining. Behavioral tests, namely tape removal and cylinder tests, were performed for assessing sensorimotor function.

In the 6h SD protocol, no significant difference ($P > 0.05$) was found either in infarct size (42.5 ± 30.4 mm^3 in sleep deprived animals vs. 44.5 ± 20.5 mm^3 in controls, mean ± s.d.), in brain swelling (10.2 ± 3.8 % in sleep deprived animals vs. 11.3 ± 2.0 % in controls) or in number of TUNEL-positive cells (21.7 ± 2.0/mm^2 in sleep deprived animals vs. 23.0 ± 1.1/mm^2 in controls). In contrast, 12h sleep deprivation increased infarct size by 40 % (82.8 ± 10.9 mm^3 in SD group vs. 59.2 ± 13.9 mm^3 in control group, $P = 0.008$) and number of TUNEL-positive cells by 137 % (46.8 ± 15/mm^2 in SD group vs. 19.7 ± 7.7/mm^2 in control group, $P = 0.003$). There was no significant difference ($P > 0.05$) in brain swelling (12.9 ± 6.3 % in sleep deprived animals vs. 11.6 ± 6.0 % in controls). The SDis protocol also increased infarct size by 76 % (3 x 12h SD 58.8 ± 20.4 mm^3 vs. no SD 33.8 ± 6.3 mm^3, $P = 0.017$) and number of TUNEL-positive cells by 219 % (32.9 ± 13.2/mm^2 vs. 10.3 ± 2.5/mm^2, $P = 0.008$). Brain swelling did not show any difference between the two groups (24.5 ± 8.4 % in SD group vs. 16.7 ± 8.9 % in control group, $p > 0.05$). Both behavioral tests did not show any concluding results.

In summary, we demonstrate that sleep deprivation aggravates brain damages in a rat model of stroke. Further experiments are needed to unveil the mechanisms underlying these effects.

1. INTRODUCTION

Stroke is one of the leading causes of disability in high-income countries and of death worldwide[1]. According to the World Health Organization, a stroke is defined as "rapidly developing clinical signs of focal disturbance of cerebral function, lasting more than 24 hours or leading to death with no apparent cause other than that of vascular origin"[2]. Stroke is more prevalent in men than in women[3] and the mean age of stroke patient is 69.8 years for men and 74.8 years for women[4]. Ischemic stroke is the consequence of a transient or permanent reduction in cerebral blood flow and affects specifically the territory of a major brain artery. The reduction in blood flow is due, in the majority of cases, to either an embolus or a local thrombosis that occludes a cerebral artery[5].

There are only few medical interventions available. Among these, intravenous thrombolysis with alteplase is the most effective treatment in the acute phase (within 4.5 hours)[6]. Patients that are not eligible for the intravenous thrombolysis may be candidates for intra-arterial thrombolysis if they are evaluated within 6 hours of symptoms. For both of these treatments, the major risk is symptomatic brain hemorrhage[7]. Unfortunately, functional recovery is, for most of the time, incomplete and therefore it is of great importance to find ways to improve stroke treatment.

Several risk factors exist and can be classified depending on whether it is modifiable or not. Nonmodifiable risk factors comprise age, ethnicity, sex, low birth weight, and family history of stroke. Modifiable risk factors include cardiovascular disease, hypertension, cigarette smoking, diabetes, carotid artery stenosis, atrial fibrillation, dyslipidemia, poor diet, obesity, physical inactivity, and postmenopausal hormone therapy[3]. Another risk factor, that is often underestimated but nevertheless important, is sleep-wake disturbances.

Sleep-wake disturbances (SWD) are frequently observed in stroke patients, with a prevalence of about 20 to 40 %. Excessive daytime sleepiness, increased sleep needs (hypersomnia) and insomnia are the most frequent ones. Several causes are involved in SWD. Brain damage itself can disrupt mechanisms underlying the regulation of sleep-wake cycles. Furthermore, consequences of stroke, such as pain, increased bed rest, stress, hypoxia or hospital environment itself may also influence sleep. SWD have been shown to have adverse consequences on cognitive and neurological functions, and finally on quality of life[8]. Many research groups have evaluated the effects of stroke on sleep with the use of electroencephalogram (EEG). Several studies have reported augmented wakefulness after

sleep onset[9-12], reduced sleep efficiency[9-13], reduced total sleep time[11-13], decreased NREM sleep stage 2 and slow-wave sleep[11 13]. It is therefore of great importance to recognize and treat, when possible, these sleep disturbances in stroke patients.

Stroke induces a cascade of mechanisms that account for brain damage, such as energy failure, excitotoxicity, peri-infarcts depolarizations, inflammation and apoptosis[5 14]. Reduction of cerebral blood flow diminishes the delivery of substrates, like oxygen or glucose, necessary for the tissue surviving. In the absence of energy, ionic gradients are not maintained, membrane potential is lost and subsequently leads to depolarization of neurons. Glutamate is released, activates its specific receptor, which allows Ca^+ and Na^+ ions to enter into the cell. Water follows passively Na^+ ions into the cell and oedema develops. Further to the increase of intracellular Ca^+ and Na^+, K^+ and glutamate diffuse in the extracellular space and generate waves of depolarization called "peri-infarcts depolarizations". The overload of intracellular Ca^+ activates several enzymes, such as proteases or lipases, which trigger cellular membrane degradation and free radicals production. The latter activate inflammatory mediators, which lead to leukocytes infiltration and to microglial activation. Free radicals also induce mitochondrial and DNA damage, which then induce apoptosis.

Sleep deprivation studies have shown several roles of sleep. For example, Magistretti's group revealed effects of sleep deprivation on glycogen metabolism in cerebral cortex of mice. They found increased expression of glucose transporter 1 (Glut1) and protein targeting to glycogen (PTG) genes, as well as enhanced glycogen synthase activity (GSynt). These results indicate an increased glucose entry into the brain and augmented glycogen synthesis. As glycogen is, for the most part, localized in astrocytes, it has been postulated that these cells are involved in the regulation of brain energy metabolism during sleep-wake cycle[15 16]. Another consequence of sleep deprivation was shown by Vgontzas and co-workers. They studied effects of mild chronic sleep restriction in young and healthy subjects. They have found, among others, that sleep deprivation increased secretion of pro-inflammatory cytokines, in particular IL-6 and TNF-α[17]. Some evidence indicates that neurotransmitter activities are also influenced by sleep. Indeed, homeostasis of extracellular glutamate is regulated by sleep-wake cycles. During waking and REM sleep, glutamate levels increased, and during NREM sleep it progressively decreased[18]. Taken together, these studies suggest an effect of sleep on several brain mechanisms like energy metabolism, cytokine production and neurotransmitter activities.

Up to now, there is a lack of knowledge about SD and stroke. The purpose of this study was to investigate, in a rat model of focal cerebral ischemia, the effects of three protocols of sleep

deprivation on the stroke-induced brain damage. Part of results obtained from this study has recently been published[19].

2. MATERIALS AND METHODS

2.1 Animals

Adult male Sprague-Dawley (Harlan, Netherlands) rats weighing 300-365 g at time of surgery were used. They were housed in Plexiglas cages in a room maintained on a 12-hour light/dark cycle (light from 9:00 a.m. to 9:00 p.m.). Ambient temperature was 22 ± 0.5°C. They had free access to food and water. All experiments were conducted with governmental approval according to local guidelines (Kantonales Veterinäramt Zürich, Switzerland) for the care and use of laboratory animals.

2.2 Surgical procedure

Focal cerebral ischemia was induced by coagulating the distal middle cerebral artery (MCA) using a procedure modified from Tamura et al.[20]. Rats were anesthesized with 2% Isoflurane (30 % O_2, remainder N_2O). A 2-cm skin incision was performed midway between the ear and eye. The skin is retracted and the parotid gland seen. The temporalis muscle was divided vertically and the skull exposed where the frontal bone joins the temporal bone. A 5x5 mm area of the bone overlying MCA was thinned with a driller and removed. The dura was delicately opened with a fine needle and then retracted. The MCA and its three main branches dorsal to the rhinal fissure were occluded by bipolar electrocoagulation. The incision was closed with silk suture. Rectal temperature was maintained between 36.5 ± 0.5°C by a warm lamp during the entire surgery. Animals were placed back into their cages once they were awake from anesthesia.

2.3 Sleep deprivation protocols (Fig 1)

2.3.1 6h SD

In a first set of experiments, 4 rats were subjected to a six-hour sleep deprivation 24 hours after surgery (Fig. 1-A). This was performed by gentle handling (knocking at the cage, giving new nesting material, introducing new objects to play with) and under continuous visual observation. Two rats were sleep deprived at the same time (each of them in an individual cage) and this occurred during light phase (start between 10:00-11:00 a.m.). Control rats (n = 4) were subjected to the same ischemia surgery but left undisturbed afterward. After sleep deprivation rats were decapitated after brief isoflurane anesthesia. Brains were removed, immediately frozen on dry ice and stored at – 80°C. Control rats were decapitated at the same time point as sleep deprived rats.

2.3.2 12h SD

In a second set of experiments, 6 rats were subjected to a 12-hour sleep deprivation 12 hours after surgery (Fig. 1-B). In the control group 6 rats were left undisturbed after induced ischemia. Rats were sacrificed and brains collected as explained in the 6h SD protocol.

2.3.3 Sleep disturbance (SDis)

In a third set of experiments, 6 rats were subjected to three times 12-hour sleep deprivation (each separated from 12-hour intervals) 12 hours after surgery (Fig. 1-C). Behavioral tests were performed to evaluate sensorimotor deficit after induced cerebral ischemia. They were performed 12h (i.e. just before sleep deprivation), 24h (i.e. just after sleep deprivation), 60h (i.e. just before sleep deprivation) and 72h (i.e. just after sleep deprivation) after surgery. Behavioral tests consisted of a session of cylinder test immediately followed by a first session of tape removal test and 40 +/- 10 minutes later by a second one. Controls rats (n = 6) were subjected to the same ischemia surgery and behavioral tests (i.e. 12h, 24h, 60h, and 72h after surgery) but were left undisturbed in between. At the end of experiment, rats were sacrificed and brains removed as in the first two protocols.

2.4 Cylinder test

This test assesses the motor function by showing the limb-use asymmetry during explorative activity. Rats were placed into a transparent Plexiglas cylinder (20 cm in diameter and 35 cm height) localized on a glass tabletop. A mirror was placed below and angled in a way that enables to see the rat's paws anytime. The test was recorded via the mirror by a camera with slow motion property[21]. Animals were placed for one session into the cylinder a few days before baseline recordings. Each session lasted 4 minutes. Baseline recording consisted of one session in the morning and one in the evening of the day before surgery. Then rats were tested 12 hours, 24 hours, 60 hours and 72 hours after surgery. Each morning sessions were performed at the same time (10:00 a.m. +/- 1 hour), as well as the evening sessions (06:00 p.m. +/- 1 hour). The number of forelimb contact with the cylinder wall or floor was counted during vertical or horizontal exploration activities. Then the percentage of asymmetry of each forelimb wall contacts was calculated as proposed by Biernaskie and co-workers: ([ipsilateral paw/(ipsilateral paw + contralateral paw)] * 100)[22].

2.5 Tape removal test

The tape removal test assesses both sensory and motor deficit and was performed in an adapted manner from the method described by Starkey et al.[23]. As this test was done right after the cylinder test, it was also performed in the cylinder. An adhesive tape (L x W: 1.2 x 0.8 cm) was applied to the distal-radial part of the forepaw. The label was attached to either the left of right forelimb in a random fashion, and a sense score and a motor score were evaluated. The sense score is defined by the time until the rat notices the tape on its paw (expressed by shaking of the forepaw and bringing it to the mouth). The motor score is defined by the time, once the tape has been noticed, until the rat removes the tape from its paw. Each session consisted of 6 trials and each trial lasted 3 minutes maximum. Two sessions per day of testing were performed with 40 +/- 10 minutes break in between. First, animals had two sessions for habituation a few days before experiment. Then, baseline was recorded on the morning and on the evening of the day before surgery. After surgery rats were tested at 12 hours, 24 hours, 60 hours and 72 hours.

2.6 Brain damage analysis

Coronal 20-µm cryostat sections from brain were collected at six levels (+2.70, +1.70, +0.70, -0.30, -1.30, -2.30 from Bregma[24]) for brain damage assessment. Twelve to 15 sections at each level were mounted on SuperFrost Plus slides (Menzel GmbH, Braunschweig, Germany) for histology assessments.

Nissl staining was performed to assess infarct area. This histological technique was invented by a German neuropathologist named Franz Nissl (1860 – 1919). It is a very widely used method to study morphology of neurons. It is based on the interaction of a basic dye, like cresyl violet, with DNA or RNA present in the cell. As neurons actively synthesize new proteins, a high concentration of rough endoplasmic reticulum is found in their cytoplasm and therefore, allows a specific staining[25]. First, sections were fixed for 20 min at room temperature with phosphate-buffered saline (PBS, pH 7.4) containing 4% paraformaldehyde and then washed with distilled water. Slides were then dipped successively in the following solutions: cresyl violet solution (1 g cresyl violet acetate in 100 ml dH_2O containing 0.25 ml glacial acetic acid) for 2 min, distilled water (30 sec), 70% ethanol (30 sec), 90% ethanol (30 sec), 95% ethanol (30 sec), 100% ethanol (30 sec), xylol (2 x 2 min). Finally, sections were covered. On digitized cresyl violet slides (Figure 2), infarct area was delineated and measured with the NIH imageJ software (NIH, Bethesda, MD, USA) and infarct volume was converted with the known distance between each of the chosen levels and eventually corrected for oedema by multiplying the ratio of the contralateral to ipsilateral volume[26]. Brain edema was calculated according to the formula from Kotani et al.: (infarct volume + ipsilateral undamaged volume – contralateral volume) x 100/contralateral volume (%)[27].

Terminal deoxynucleotidyl *t*ransferase mediated d*U*TP *n*ick *e*nd *l*abeling (TUNEL) staining was used for detection and quantification of apoptosis at the single-cell level. The principal feature of apoptosis is DNA fragmentation. These DNA breaks can be detected by labeling free 3'OH ends with modified nucleotides (like biotin-dUTP, digoxigenin-dUTP or fluorescein-dUTP) by the mean of an enzyme named terminal deoxynucleotidyl transferase (TdT)[28 29]. In our case, we used the *In Situ* Cell Death Detection Kit, Fluorescein (Roche, Basel, Switzerland) and visualized sections by fluorescence microscopy. Fluorescein is a green dye, which has an excitation wavelength at 494 nm and an emission wavelength at 521 nm. Sections were first fixed with 4% paraformaldehyde and then washed 3 x 5 minutes with PBS. After 2 minutes incubation on ice in permeabilisation solution P1 (1 g NaCitrat in 1000

ml PBS + 1 ml Triton X-100, pH 7.4), slides were immersed in 200 ml 0.1 M Citrat Buffer P2 (pH 6.0) and irradiated 1 minute with 750 W high microwave. For rapid cooling, 80 ml double distilled water at room temperature was applied. Sections were then transferred into PBS at room temperature before 30 minutes immersion in blocking solution Buffer A (2 ml NGS 100 %, 200 µl Gelatine, 60 µl Triton X-100, 200 µg bovine serum albumin (BSA), 17.540 ml PBS). 50 µl of TUNEL reaction mixture were applied to each section for 60 minutes incubation at 37 °C in a humidified atmosphere in the dark. Then slides were rinsed 3 times in PBS for 5 minutes each. Sections were finally counterstained with 4',6-diamidino-2-phenylindole (DAPI) which has a blue emission (excitation wavelength 358 nm (ultraviolet), emission wavelength 461 nm). DAPI was applied for 3 to 5 minutes before covering slides. Sections were then evaluated under a fluorescent microscope and the number of TUNEL positive cells was counted in the injured area measuring 20 mm^2 (Figure 3).

2.7 Statistics

Data were presented as mean ± standard deviation (s.d.). The significance of differences in means of infarct volume, number of TUNEL positive cells and brain swelling was assessed by independent *t*-test. Tape removal and cylinder tests were evaluated by repeated-measures ANOVA (SPSS, 12.02 for Windows) followed by post-hoc independent *t*-test. The significance level was set at P values < 0.05.

3. RESULTS

3.1 6-hour sleep deprivation protocol

Brain damage induced by MCA occlusion was confined to primary sensorimotor cortex. No significant difference (P = 0.9) in infarct volume was found between the two groups. Infarct size was 42.5 ± 30.4 mm^3 (mean ± s.d.) in sleep deprived animals vs. 44.5 ± 20.5 mm^3 in controls (Figure 4 A).

TUNEL staining for cell death showed 21.7 ± 2.0/mm^2 damaged cells in SD group vs. 23.0 ± 1.1/mm^2 in control group (Figure 4 B). This difference was not significant (P = 0.3).

Brain swelling was 10.2 ± 3.8 % in sleep deprived animals vs. 11.3 ± 2.0 % in controls (Figure 4 C). Here again there was no significant difference (P = 0.6) between the two groups.

3.2 12-hour sleep deprivation protocol

Cresyl violet staining showed a significantly (P = 0.008) increased infarct size in the sleep deprivation group (82.8 ± 10.9 mm^3 in sleep deprived animals vs. 59.2 ± 13.9 mm^3 in controls) (Figure 4 A). Infarct size was increased by 40 %.

The number of TUNEL-positive cells was significantly (P = 0.003) increased in the SD group (46.8 ± 15/mm^2) compared with the control group (19.7 ± 7.7/mm^2) (Figure 4 B). The number of damaged cells was increased by 137 %.

No significant difference (P = 0.7) in brain swelling was seen between the two groups. Swelling was 12.9 ± 6.3 % in sleep deprived animals vs. 11.6 ± 6.0 % in controls (Figure 4 C).

3.3 Sleep disturbance protocol

With this protocol, there was a significant difference (P = 0.017) for the infarct size between the sleep deprived group (58.8 ± 20.4 mm^3) and the control group (33.8 ± 6.3 mm^3) (Figure 4 A). The infarct size was increased by 76 %.

TUNEL positive staining also showed a significant (P = 0.008) increase by 219 % in the number of damaged cells in the sleep deprivation group (32.9 ± 13.2/mm^2) compared with the control group (10.3 ± 2.5/mm^2) (Figure 4 B).

There was no significant difference (P = 0.1) concerning swelling between the two groups (24.5 ± 8.4 % in sleep deprived group vs. 16.7 ± 8.9 % in control group) (Figure 4 C).

Results for the cylinder test showed symmetrical use of the forelimbs before ischemia. However, after surgery rats from the sleep deprivation group used preferentially their unaffected paw for postural support (Figure 5). Repeated-measures ANOVA verified an effect of time by group interaction ($F_{4, 40}$ = 6.399, P < 0.001) and post-hoc independent *t*-test revealed significant differences at 12h, 24h and 60h after surgery.

Tape removal test revealed deficit after ischemia and sleep deprivation in the sense score but not in the motor score. The sense score of sleep-deprived rats was significantly higher for the affected forelimb. Indeed, sleep-deprived animals took significantly longer than the control group to sense the tape when placed on their affected paw. This impairment persisted throughout the whole experiment (repeated-measures ANOVA, time * group, $F_{4,40}$ = 12.677,

P = 0.008). Post-hoc independent *t*-test revealed significant differences at 12h, 24h, 60h and 72h after surgery. There was no significant difference (P = 0.17) for the sense score in the unaffected (left) paw. For the motor score in both left and right paws there was no significant difference (P > 0.05). Although the affected (right) motor score showed the tendency to be higher for the sleep-deprived group, repeated-measures ANOVA could not verify an effect of time by group.

4. DISCUSSION

In this study, we evaluated effects of three SD protocols on stroke-induced brain damage in the rat. Two protocols, i.e. 12h SD and SDis, induced an increase in infarct volume and number of TUNEL positive cells. In the 12h SD protocol, infarct volume was increased by 40 % and number of TUNEL positive cells was augmented by 137 %. In the SDis protocol infarct volume was enhanced by 76 % and number of TUNEL positive cells was increased by 219 %. This is the first direct demonstration of a negative effect of sleep deprivation on stroke pathology.

In the 6h SD protocol there was no significant difference between the two groups either for infarct size or for cell damage. This might be due to two factors in comparison with the 12h SD protocol, i.e. the time window chosen for SD and the duration of SD. In the 6h SD protocol, SD was started 24 hours after ischemia compared with 12 hours in the 12h SD protocol. It has been shown that cells in (or near) the ischemic area are most vulnerable within 24 hours after onset of ischemia. For example, Garcia et al found major ischemic alterations like neuronal swelling, scalloping and neuronal shrinkage until 24 hours post-ischemia[30]. In addition, the duration of SD may also play a role. The 6h SD protocol suggest that sleep deprivation as short as 6 hours may be not sufficient to have any influence at the brain cellular level.

We attempted to use the SDis protocol to assess effects of sleep-wake disturbances on stroke evolution. Increased sleep needs, insomnia or excessive daytime sleepiness are examples of sleep-wake disturbances, which frequently occur after stroke. Sleep and wakefulness are regulated by structures in the thalamus, brainstem and posterior hypothalamus. It is therefore possible that stoke may lead to such deregulations[31]. Indeed, our results suggest that sleep disturbances during the acute phase of ischemia may have negative impact on stroke.

Many data indicate possible effects of sleep on mechanisms involved in stroke. Ischemic stroke is due to a sudden reduction of blood flow, which triggers a series of events (called ischemic cascade) including energy failure, excitotoxicity, free radical generation and inflammation[5 14]. Short-term sleep deprivation has been shown to affect energy metabolism in the brain. Indeed, the level of glucose transporter 1 (Glut 1) gene is enhanced and the activity of glycogen synthase (GSynt) increased. This allows an augmented entry of glucose into brain parenchyma and favors glycogen synthesis[15 16]. Other studies reveal increasing brain energy consumption after sleep deprivation. Kong et al demonstrated decreasing amount of glycogen in gray and white matter of rat brains with sleep deprivation and increasing amount with recovery sleep[32]. Vyazovskiy and co-workers evaluated cortical metabolic rates in mice by 2-deoxyglucose-uptake measurements: 2-deoxyglucose-uptake decreased after sleep and increased when mice where kept awake[33]. Short-term sleep deprivation also increases the extracellular glutamate concentration[18], which could contribute to worsen excitotoxicity. Some evidence indicates that long-term sleep deprivation or sleep restriction increases secretion of pro-inflammatory cytokines such as IL-6, TNF-α and soluble TNF-α receptor I (sTNF-αRI)[17 34]. These cytokines are important mediators in the inflammatory phase of cerebral ischemia[35]. Several studies have shown that prolonged sleep deprivation decreases levels of glutathione and superoxide dismutase, two oxidative stress parameters[36-38]. Glutathione protects cells against damage induced by free radicals. When its level diminishes, equilibrium between reactive oxygen species (ROS) and antioxydant defense mechanisms is lost[36]. These results suggest that sleep deprivation may induce oxidative stress in brain and by doing so could intensify cellular damage in the ischemia site. Taken together, these data show that sleep deprivation could aggravate stroke through exacerbation of its pathophysiological mechanisms. However, further studies are needed to characterize this functioning.

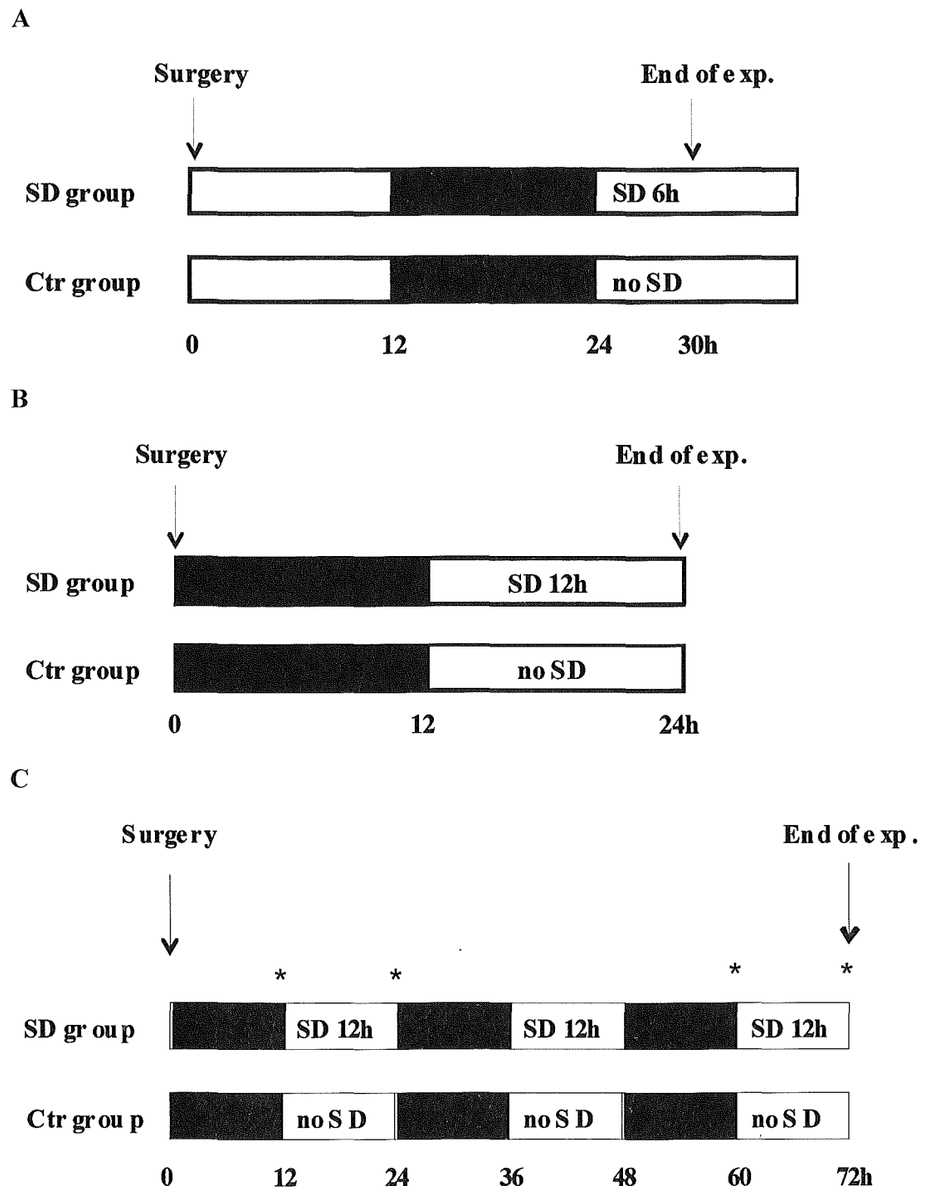
Effects of SD on sensorimotor function were evaluated by the mean of two behavioral tests. Both tests revealed functional deficits in the affected paw after ischemia and there was also group difference. However, these results do not indicate any effect of SD since the group difference was already present at 12 hours after ischemia before SD was carried out (Fig. 5 and 6). As individual difference in both tests was big and the number of animals used in this study was relatively small, further experiments with a larger number of animals are needed to draw a conclusion about the effects of SD on functional outcomes.

In conclusion, this study demonstrates that sleep deprivation occurring early after stroke, aggravates brain damage. Thus, improving sleep quality of stroke patients may enhance their chance of better outcome.

ANNEXE I:

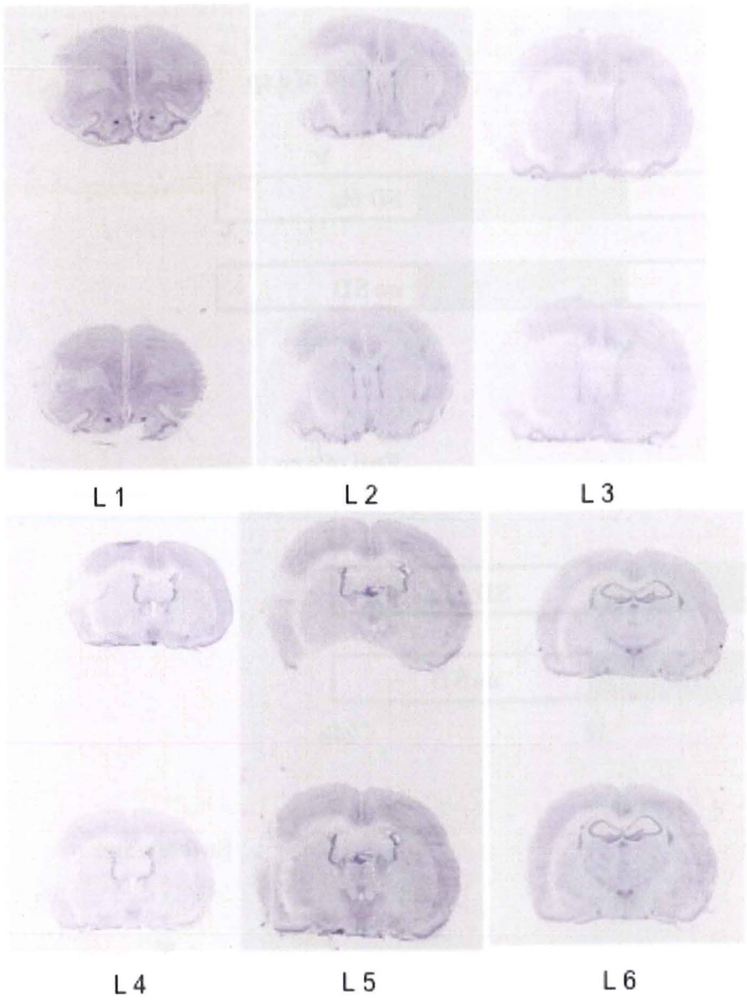
Figures 1 - 6

Figure 1



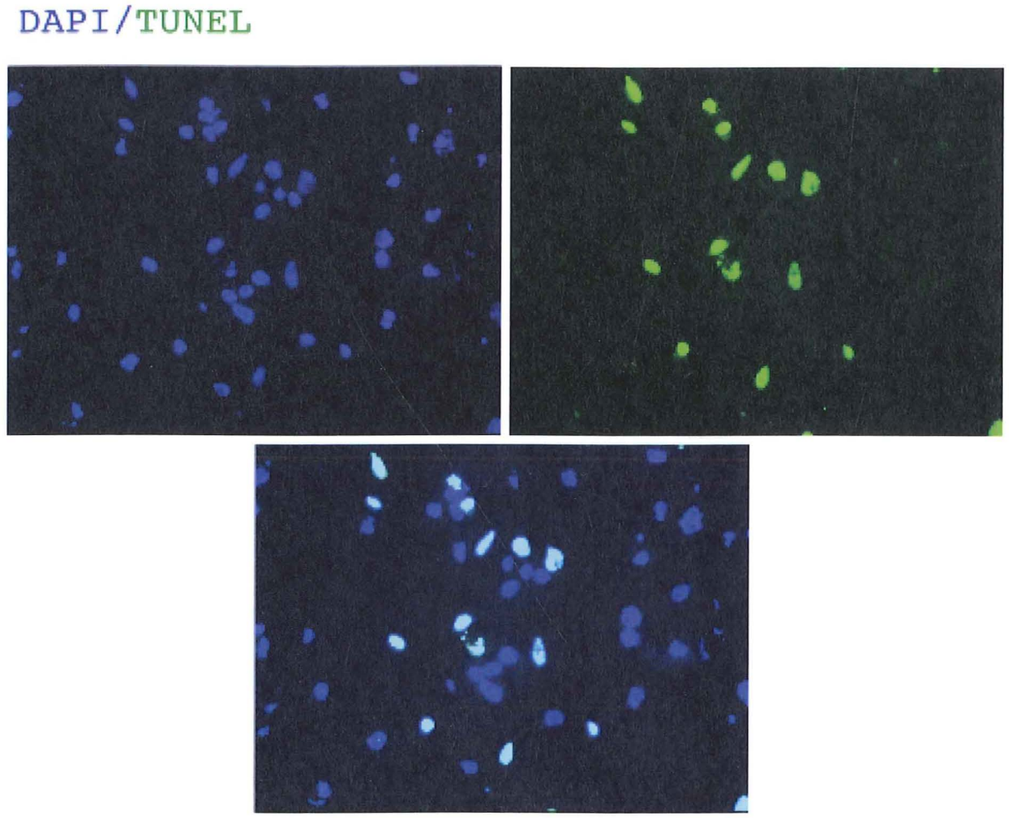
Sleep deprivation (SD) protocols: 6h SD (A), 12h SD (B), sleep disturbance (C). The white and black bars indicate the light and dark period, respectively. (C) asterisks represent behavioral tests. (Adapted from Gao et al.[19]).

Figure 2



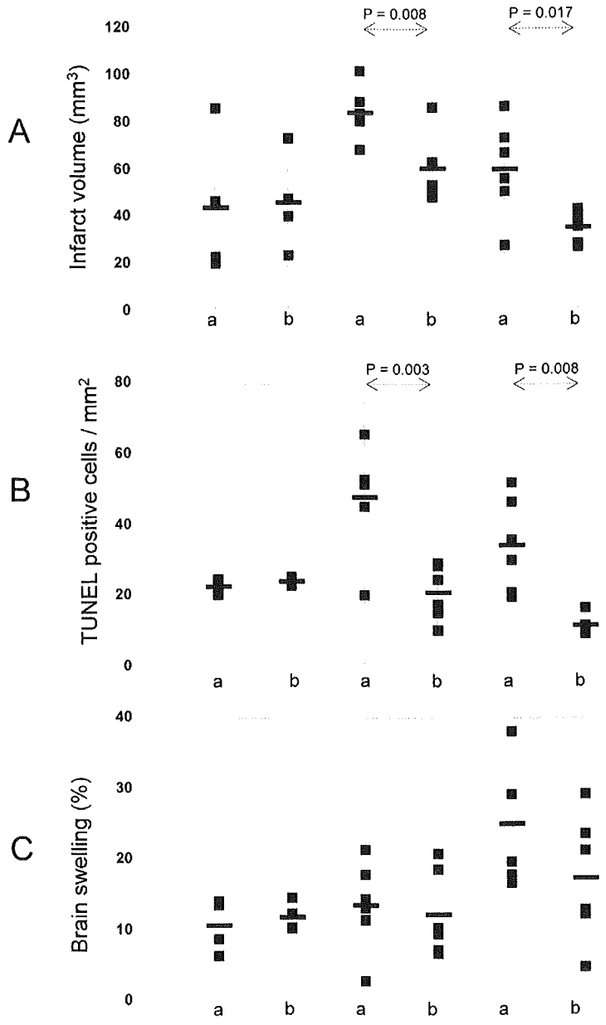
Nissl Staining: coronal 20-µm cryostat sections from brain collected at six levels (L1 to L6 corresponding to +2.70, +1.70, +0.70, -0.30, -1.30, -2.30 from Bregma). The non stained area corresponds to the lesion, which is limited to the somatosensory cortex.

Figure 3



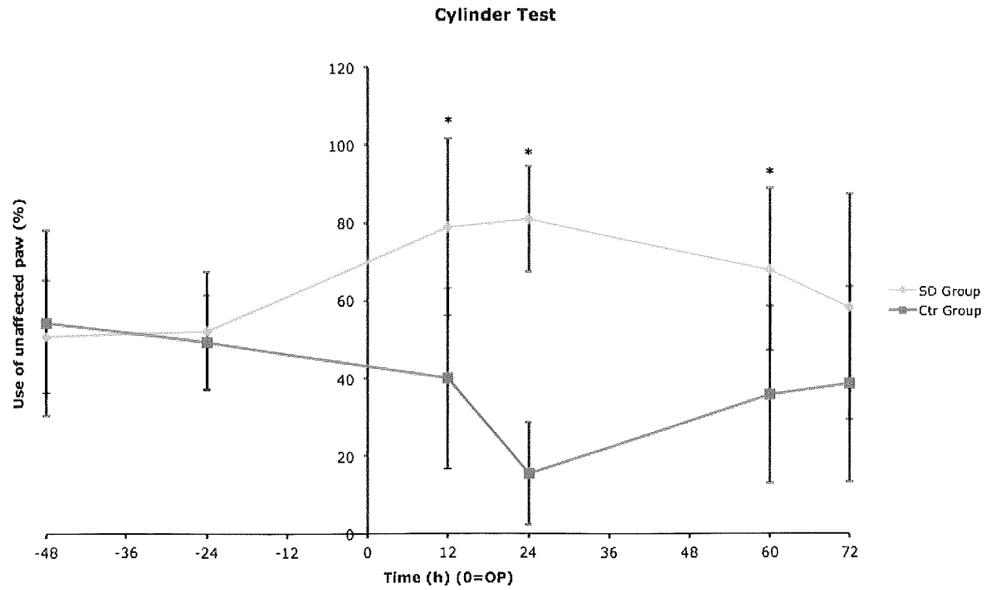
Picture of immunofluorescent stainings of the infarct lesion. 4',6-diamidino-2-phenylindole (DAPI) staining colours nuclei of all cells in blue (upper left panel). Terminal transferase biotinylated-dUTP nick end labeling (TUNEL) staining (green dye) was used to highlights apoptotic cells (upper right panel). The down panel represents the double staining DAPI / TUNEL.

Figure 4



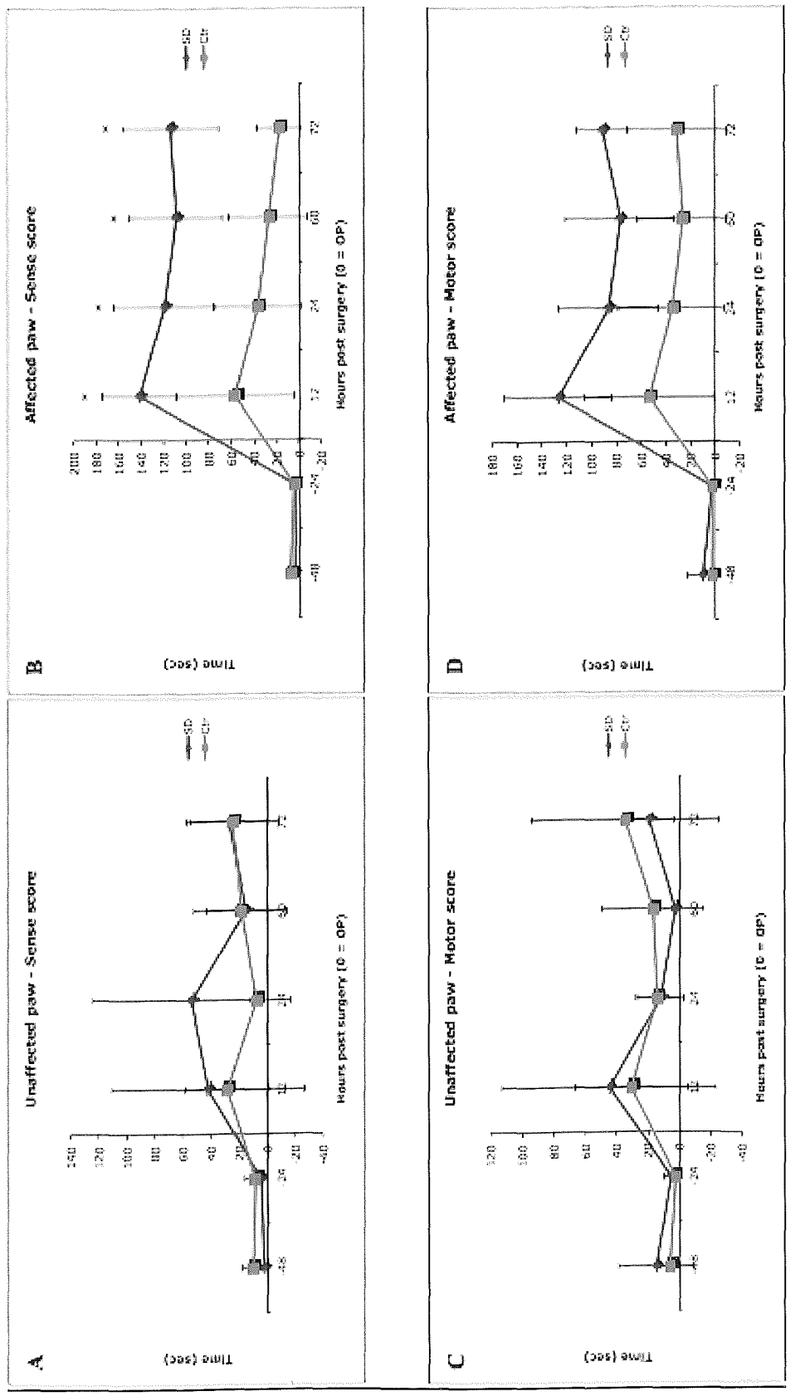
Effects of the 3 different sleep deprivation (SD) protocols on infarct volume (A), number of TUNEL positive cells (B), and brain swelling (C) in individual animals. Horizontal bars represent the mean values. Independent *t*-test for each experiment was used for the statistical analysis. a, ischemia with sleep deprivation. b, ischemia without sleep deprivation. Picture adapted from Gao et al.[19].

Figure 5



Cylinder test. Percent use of unaffected paw during exploratory activity in a cylinder of sleep deprivation (SD) and control (Ctr) group before and after middle cerebral artery occlusion. Data are mean ± s.d. (asterisks denote significant difference from Ctr group, $P < 0.001$, repeated-measures ANOVA, independent *t*-test).

Figure 6



Tape removal test. (A, B) show sense scores for the unaffected and affected forepaws, respectively. After ischemia rats of the sleep deprivation (SD) group took significantly longer to sense the sticky tape on their affected paw (B). No significant differences between SD and control (Ctr) group were observed in the sense score of the unaffected paw. (C, D) Show the motor score of the left and right forepaws, respectively. No significant differences between SD and Ctr groups were observed in motor scores for both paws. Data are means ± s.d. (asterisks denote significant differences from control group ; P = 0.008, repeated measures ANOVA, independent *t*-test).

REFERENCES

1. Lopez AD, Mathers CD, Ezzati M, Jamison DT, Murray CJL. Global and regional burden of disease and risk factors, 2001: systematic analysis of population health data. *The Lancet* 2006;367(9524):1747-57.
2. Hatano S. Experience from a multicentre stroke register: a preliminary report. *Bull World Health Organ* 1976;54(5):541-53.
3. Goldstein LB, Adams R, Alberts MJ, Appel LJ, Brass LM, Bushnell CD, et al. Primary prevention of ischemic stroke: a guideline from the American Heart Association/American Stroke Association Stroke Council: cosponsored by the Atherosclerotic Peripheral Vascular Disease Interdisciplinary Working Group; Cardiovascular Nursing Council; Clinical Cardiology Council; Nutrition, Physical Activity, and Metabolism Council; and the Quality of Care and Outcomes Research Interdisciplinary Working Group: the American Academy of Neurology affirms the value of this guideline. *Stroke* 2006;37(6):1583-633.
4. Feigin VL, Lawes CMM, Bennett DA, Anderson CS. Stroke epidemiology: a review of population-based studies of incidence, prevalence, and case-fatality in the late 20th century. *The Lancet Neurology* 2003;2(1):43-53.
5. Dirnagl U, Iadecola C, Moskowitz MA. Pathobiology of ischaemic stroke: an integrated view. *Trends Neurosci* 1999;22(9):391-7.
6. Hacke W, Kaste M, Bluhmki E, Brozman M, Davalos A, Guidetti D, et al. Thrombolysis with alteplase 3 to 4.5 hours after acute ischemic stroke. *N Engl J Med* 2008;359(13):1317-29.
7. Adams HP, Jr., del Zoppo G, Alberts MJ, Bhatt DL, Brass L, Furlan A, et al. Guidelines for the early management of adults with ischemic stroke: a guideline from the American Heart Association/American Stroke Association Stroke Council, Clinical Cardiology Council, Cardiovascular Radiology and Intervention Council, and the Atherosclerotic Peripheral Vascular Disease and Quality of Care Outcomes in Research Interdisciplinary Working Groups: the American Academy of Neurology affirms the value of this guideline as an educational tool for neurologists. *Stroke* 2007;38(5):1655-711.
8. Bassetti CL. Sleep and stroke. *Semin Neurol* 2005;25(1):19-32.
9. Gottselig JM, Bassetti CL, Achermann P. Power and coherence of sleep spindle frequency activity following hemispheric stroke. *Brain* 2002;125(Pt 2):373-83.

10. Muller C, Achermann P, Bischof M, Nirkko AC, Roth C, Bassetti CL. Visual and spectral analysis of sleep EEG in acute hemispheric stroke. *Eur Neurol* 2002;48(3):164-71.
11. Terzoudi A, Vorvolakos T, Heliopoulos I, Livaditis M, Vadikolias K, Piperidou H. Sleep Architecture in Stroke and Relation to Outcome. *European Neurology* 2009;61(1):16-22.
12. Vock J, Achermann P, Bischof M, Milanova M, Muller C, Nirkko A, et al. Evolution of sleep and sleep EEG after hemispheric stroke. *J Sleep Res* 2002;11(4):331-8.
13. Bassetti CL, Aldrich MS. Sleep electroencephalogram changes in acute hemispheric stroke. *Sleep Med* 2001;2(3):185-94.
14. Durukan A, Tatlisumak T. Acute ischemic stroke: Overview of major experimental rodent models, pathophysiology, and therapy of focal cerebral ischemia. *Pharmacology Biochemistry and Behavior* 2007;87(1):179-97.
15. Petit JM, Tobler I, Allaman I, Borbely AA, Magistretti PJ. Sleep deprivation modulates brain mRNAs encoding genes of glycogen metabolism. *Eur J Neurosci* 2002;16(6):1163-7.
16. Petit JM, Tobler I, Kopp C, Morgenthaler F, Borbely AA, Magistretti PJ. Metabolic response of the cerebral cortex following gentle sleep deprivation and modafinil administration. *Sleep* 2010;33(7):901-8.
17. Vgontzas AN. Adverse Effects of Modest Sleep Restriction on Sleepiness, Performance, and Inflammatory Cytokines. *Journal of Clinical Endocrinology & Metabolism* 2004;89(5):2119-26.
18. Dash MB, Douglas CL, Vyazovskiy VV, Cirelli C, Tononi G. Long-Term Homeostasis of Extracellular Glutamate in the Rat Cerebral Cortex across Sleep and Waking States. *Journal of Neuroscience* 2009;29(3):620-29.
19. Gao B, Cam E, Jaeger H, Zunzunegui C, Sarnthein J, Bassetti CL. Sleep disruption aggravates focal cerebral ischemia in the rat. *Sleep* 2010;33(7):879-87.
20. Tamura A, Graham DI, McCulloch J, Teasdale GM. Focal cerebral ischaemia in the rat: 1. Description of technique and early neuropathological consequences following middle cerebral artery occlusion. *J Cereb Blood Flow Metab* 1981;1(1):53-60.
21. Schallert T, Fleming SM, Leasure JL, Tillerson JL, Bland ST. CNS plasticity and assessment of forelimb sensorimotor outcome in unilateral rat models of stroke, cortical ablation, parkinsonism and spinal cord injury. *Neuropharmacology* 2000;39(5):777-87.
22. Biernaskie J. Efficacy of Rehabilitative Experience Declines with Time after Focal Ischemic Brain Injury. *Journal of Neuroscience* 2004;24(5):1245-54.

23. Starkey ML, Barritt AW, Yip PK, Davies M, Hamers FP, McMahon SB, et al. Assessing behavioural function following a pyramidotomy lesion of the corticospinal tract in adult mice. *Exp Neurol* 2005;195(2):524-39.
24. Paxinos G, Watson C. The rat brain in stereotaxic coordinates. 2007.
25. Kadar A, Wittmann G, Liposits Z, Fekete C. Improved method for combination of immunocytochemistry and Nissl staining. *J Neurosci Methods* 2009;184(1):115-8.
26. Junge CE. The contribution of protease-activated receptor 1 to neuronal damage caused by transient focal cerebral ischemia. *Proceedings of the National Academy of Sciences* 2003;100(22):13019-24.
27. Kotani Y, Morimoto N, Oida Y, Tamura Y, Tamura S, Inoue T, et al. Prevention of in vitro and in vivo acute ischemic neuronal damage by (2S)-1-(4-amino-2,3,5-trimethylphenoxy)-3-{4-[4-(4-fluorobenzyl) phenyl]-1-piperazinyl}-2-propanol dimethanesulfonate (SUN N8075), a novel neuroprotective agent with antioxidant properties. *Neuroscience* 2007;149(4):779-88.
28. Gavrieli Y, Sherman Y, Ben-Sasson SA. Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. *J Cell Biol* 1992;119(3):493-501.
29. Sgonc R, Gruber J. Apoptosis detection: an overview. *Exp Gerontol* 1998;33(6):525-33.
30. Garcia JH, Yoshida Y, Chen H, Li Y, Zhang ZG, Lian J, et al. Progression from ischemic injury to infarct following middle cerebral artery occlusion in the rat. *Am J Pathol* 1993;142(2):623-35.
31. Bassetti CL, Hermann DM. Sleep and stroke. *Handbook of Clinical Neurology* 2011;Vol. 99 (3rd series)(Sleep Disorders, Part 2):1051 - 72.
32. Kong J, Shepel PN, Holden CP, Mackiewicz M, Pack AI, Geiger JD. Brain glycogen decreases with increased periods of wakefulness: implications for homeostatic drive to sleep. *J Neurosci* 2002;22(13):5581-7.
33. Vyazovskiy VV, Cirelli C, Pfister-Genskow M, Faraguna U, Tononi G. Molecular and electrophysiological evidence for net synaptic potentiation in wake and depression in sleep. *Nature Neuroscience* 2008;11(2):200-08.
34. Shearer WT, Reuben JM, Mullington JM, Price NJ, Lee BN, Smith EO, et al. Soluble TNF-alpha receptor 1 and IL-6 plasma levels in humans subjected to the sleep deprivation model of spaceflight. *J Allergy Clin Immunol* 2001;107(1):165-70.
35. Mergenthaler P, Dirnagl U, Meisel A. Pathophysiology of stroke: lessons from animal models. *Metab Brain Dis* 2004;19(3-4):151-67.

36. D'Almeida V, Lobo LL, Hipolide DC, de Oliveira AC, Nobrega JN, Tufik S. Sleep deprivation induces brain region-specific decreases in glutathione levels. *Neuroreport* 1998;9(12):2853-6.
37. Ramanathan L, Gulyani S, Nienhuis R, Siegel JM. Sleep deprivation decreases superoxide dismutase activity in rat hippocampus and brainstem. *Neuroreport* 2002;13(11):1387-90.
38. Singh R, Kiloung J, Singh S, Sharma D. Effect of paradoxical sleep deprivation on oxidative stress parameters in brain regions of adult and old rats. *Biogerontology* 2008;9(3):153-62.

ANNEXE II:

Article published in Sleep:

Gao B, Cam E, Jaeger H, Zunzunegui C, Sarnthein J, Bassetti CL. Sleep disruption aggravates focal cerebral ischemia in the rat. *Sleep* 2010;33(7):879-87.

Sleep Disruption Aggravates Focal Cerebral Ischemia in the Rat

Bo Gao, MD[1]; Ertugrul Cam, DVM[1]; H. Jaeger[1]; C. Zunzunegui, MD[1]; Johannes Sarnthein, PhD[3]; Claudio L. Bassetti, MD[1,2]

[1]Department of Neurology, University Hospital Zürich, Switzerland; [2]Neurocenter of Southern Switzerland, Lugano, Switzerland; [3]Department of Neurosurgery, University Hospital Zürich, Zürich, Switzerland

Study Objectives: Sleep changes are frequent in stroke patients and predict a poor outcome. It remains unclear how sleep influences stroke evolution and recovery. We assessed effects of sleep disruption on brain damage and on the expression of axon sprouting genes after focal cerebral ischemia in rats.

Design: 12 h after ischemia induced by occlusion of the middle cerebral artery, rats were subjected to sleep disruption including sleep deprivation for 12h (SDpv12h) and sleep disturbances (SDis) by SDpv12h for consecutive 3 days. Control groups included ischemia without SDpv12h or SDis, sham surgery plus SDis and sham surgery without SDis. Sleep changes were evaluated based on EEG and EMG recordings.

Measurements and Results: SDpv12h increased the infarct volume by 40% (SDpv12h 82.8 ± 10.9 vs. control 59.2 ± 13.9 mm^3, P = 0.008) and SDis by 76% (SDis 58.8 ± 20.4 vs. control 33.8 ± 6.3 mm^3, P = 0.017). SDpv12h also increased the number of damaged cells, visualized by TUNEL staining, by 137% (SDpv12h 46.8 ± 15 vs. control 19.7 ± 7.7/mm^2, P < 0.001) and SDis by 219% (SDis 32.9 ± 13.2 vs. control 10.3 ± 2.5/mm^2, P = 0.002). In addition, SDis significantly elevated the expression of the axonal extension inhibitory molecule neurocan (SDis 14.3 ± 0.4 vs. control 6.2 ± 0.1-fold of change, P < 0.001) in the injured hemisphere.

Conclusions: These results provide the first direct evidence for a detrimental impact of sleep disruption on stroke evolution and suggest a potential role of sleep modulating treatments on stroke outcomes.

Keywords: Sleep deprivation, stroke, neuroplasticity, axonal sprouting gene

Citation: Gao B; Cam E; Jaeger H; Zunzunegui C; Sarnthein J; Bassetti CL. Sleep disruption aggravates focal cerebral ischemia in the rat. *SLEEP* 2010;33(7):879-887.

ISCHEMIC STROKE REMAINS ONE OF LEADING CAUSES OF DEATH IN INDUSTRIALIZED COUNTRIES AND IS ONE OF THE MOST IMPORTANT CAUSES OF long-term disability. Sleep disturbances are frequently observed in post-stroke patients and appear to have detrimental consequences on neurological deficits, rehabilitation, cognitive functions and ultimately on the quality of life.[1-3] It is unknown, however, the impact of sleep disruption on ischemia-related pathophysiology and plasticity process that determine functional outcome.

Ischemic stroke, due to a sudden reduction of blood flow, triggers a cascade of events, including energy failure, excitotoxicity, free radical generation, and inflammation.[4,5] Evidence from sleep deprivation (SDpv) experiments under healthy conditions suggests also fundamental effects of sleep on brain energy metabolism, neurotransmitter activities and production of proinflammatory molecules.[6-11]

After ischemic insults, the injured brain undergoes an extensive neuronal reconnection and reorganization.[12,13] Axonal sprouting in the peri-infarct area represents an important component of the post-stroke brain repair. A set of neuronal growth-associated genes has been identified[14] to be involved in initiating, maintaining and terminating post-stroke axonal sprouting that occurs within days after ischemic insult.[15] Our recent finding that the sleep stimulant gamma hydroxybutyrate alters expression profiles of neuroplasticity-related genes and accelerates stroke recovery[16] suggest that sleep could modulate post-stroke brain plasticity at the molecular level. Since SDpv has been found to change expression of certain plasticity-related genes in healthy rodents,[17,18] it is likely that sleep disruption after stroke could also alter expression of axonal sprouting genes.

The aim of this study was to test the hypothesis in a rat model of focal cerebral ischemia that sleep disruption could exacerbate brain damage and change expression of genes associated with axonal sprouting. We focused in this study on acute effects of sleep disruption as an initial step to understand sleep's role in modulation of stroke recovery. Sleep disruption was induced by two protocols, i.e., a short-term SDpv and a prolonged sleep disturbance (SDis) over 3 days. For gene expression assay, we chose a small set of genes whose response to stroke is within the first 3 days after insult, such as growth-promoting genes Gap43 and c-jun, and growth-inhibiting genes neurocan, ephrinA5, and ephrinB1.[14] In this study, assessments of brain damage and gene expression were conducted in the same brain.

METHODS

Animals

Male Sprague Dawley rats (n = 50), 8 weeks old and weighting 200-250g at the time of surgery, were used in this study. They were housed under 12-h light/dark cycle (light on 09:00-21:00) and ambient temperature at 22 ± 0.5°C. Food and water were provided *ad libitum*. All experiments were conducted with governmental approval according to local guidelines (Kantonales Veterinäramt Zürich, Switzerland) for the care and use of laboratory animals. Effort was made to minimize the number of animals used.

Submitted for publication August, 2009
Submitted in final revised form January, 2010
Accepted for publication January, 2010
Address correspondence to: Claudio L. Bassetti, MD, Department of Neurology/Neurocenter of Southern Switzerland, University Hospital Zürich, Frauenklinikstrasse 26, 8091 Zürich, Switzerland; Tel: 41 44 255 5503; Fax: 41 44 255 4649; E-mail: claudio.bassetti@usz.ch

Electrode Implantations, Data Acquisition, and Vigilance State Analysis

Rats were anesthetized with 2% isoflurane in 30% O_2 and 70% N_2O. Four gold-coated miniature screws (0.9 mm diameter) were inserted in the skull over both sides of the frontal cortex (Lateral to midline: 2 mm, Bregma: −2 and +2 mm) to record electroencephalogram (EEG). Two EEG electrodes on one side of the brain were paired to register EEG from each hemisphere. Two gold wires were inserted into neck muscles to record electromyogram (EMG). The electrodes were fixed to the skull with dental cement. After the surgery, rats were housed individually and allowed 7-10 days of recovery. They were then connected to recording system by a flexible cable and a swivel. Polygraphic recordings were performed with the Embla system and the SOMNOLOGICA software (Flaga, Iceland). EEG signals were sampled at 100 Hz with band-pass filter for low and high cut set at 0.3 and none, respectively, and EMG signals at 200 Hz with band-pass filter for low and high cut set at 10 and none, respectively. They were recorded for 8 h (11:00-19:00) in the light phase and 12 h (21:00-9:00) in the dark phase.

Vigilance states were scored by visual inspection of 8-s epochs. Wakefulness (W) was defined by the presence of a desynchronized EEG activity combined with elevated neck muscle tone; slow wave sleep (SWS) by high-amplitude slow wave EEG activities with a low-EMG and paradoxical sleep (PS) by dominant theta frequency activity (6–8 Hz) in EEG with low EMG signals. The EEG activity recorded on the contralateral hemisphere (i.e., undamaged) to ischemia was mainly used for scoring. Whenever there were too many artifacts, the ipsilateral channel was consulted to determine vigilance states. The percentage of each state was calculated for 8 h during the light (11:00-19:00) and 8 h during dark phase (21:00-5:00). The episode length for each vigilance states was also computed and used for assessing sleep fragmentation. To disregard brief interruptions of episodes and short episodes, we adopted minimum criteria of 12-s episode duration and 8-s episode interruption.[19]

EEG power spectra were computed from 8-s epochs recorded on the contralateral hemisphere by custom programming on the basis of standard mathematical and signal analysis functions in Matlab. We implemented the multi-taper method, which allows to trade resolution in the frequency domain for reduced variance. Spectra were calculated with a window length of 4 sec, overlap 2 sec, bandwidth parameter nw = 2 and k = 3 tapers, which offers optimal spectral smoothing. The power spectral density was given in units of 10*log 10 (μV^2/Hz). Epochs with artifacts were excluded by a threshold procedure. The percentage of artifact-free epochs during wakefulness was 70 ± 24, 65 ± 18, 64 ± 27, and 71 ± 19 for the 4 experimental groups (see *Experimental protocols*) and 86 ± 15, 82 ± 18, 80 ± 30, and 90 ± 12 during SWS. Spectral band power was computed for the frequency bands: delta (1-4 Hz), theta (4-8 Hz), sigma (11-16 Hz), and beta (16-25 Hz). Delta power during SWS was averaged for the first 2 and 8 h for the light and the dark phase, and the relative change with respect to baseline was computed for corresponding phases.

INDUCTION OF FOCAL CEREBRAL ISCHEMIA

After a 24-h baseline polygraphic recording, rats were anesthetized with 2% isoflurane (30% O_2, remainder N_2O) for the ischemia surgery that was carried out with occlusion of the distal middle cerebral artery (MCAo). The procedure was modified from Tamura et al.[20] Briefly, a 2 cm vertical skin incision is made midway between the ear and eye. The skull was exposed where the frontal bone joins the temporal bone. A 5×5 mm area of the bone overlying the middle cerebral artery (MCA) was removed and the dura retracted. The MCA and its three main branches dorsal to the rhinal fissure were occluded by bipolar electrocoagulation. The incision was closed with silk suture. Sham-control animals were subjected to the same procedure except for dura removal and vessel electrocoagulation. Rectal temperature was maintained between 36.5 ± 0.5°C by a warm lamp during the entire surgery. Animals were placed back into their home cages once they were awake from anesthesia.

Neurologic Assessments

The animal's motor behavior was carefully evaluated by a 4-point scale method described by Bederson et alcv[21] at 12 h after stroke and at the end of experiments.

Experimental Protocols and Brain Tissue Collection (Figure 1)

SDpv experiments (Figure 1A) SDpv was carried out 12 h after the ischemia during the light phase by gently knocking at the cage or providing the rats with new playing materials when they started showing signs of sleep. SDpv was carried out for either 6 h (SDpv 6h) or 12 h (SDpv12h). Control rats (ischemia/without SDpv) were subjected to the same ischemia surgery but left undisturbed afterward. Two groups in each experiment were used, i.e., ischemia/SDpv 6h (n = 4) and ischemia/without SDpv 6 h (n = 4); ischemia/SDpv 12h (n = 6) and ischemia/without SDpv12h (n = 6). At end of experiments, rats were decapitated after brief isoflurane anesthesia and brains removed, frozen immediately on dry ice and stored at −80°C. Control rats were decapitated at the same time point as SDpv rats.

SDis experiments (Figure 1B) SDpv12h was carried out as described above for 3 consecutive days during the light phase. Rats were undisturbed and allowed to sleep during the following 12-h dark phase. At the end of the experiment, i.e., 72 h after ischemia surgery and around 21:00, rats were decapitated, and brains harvested as described above. One ischemia/SDis group (n = 6) and 3 control groups (n = 6 per group) i.e., ischemic/without SDis, sham/SDis, sham/without SDis, were used.

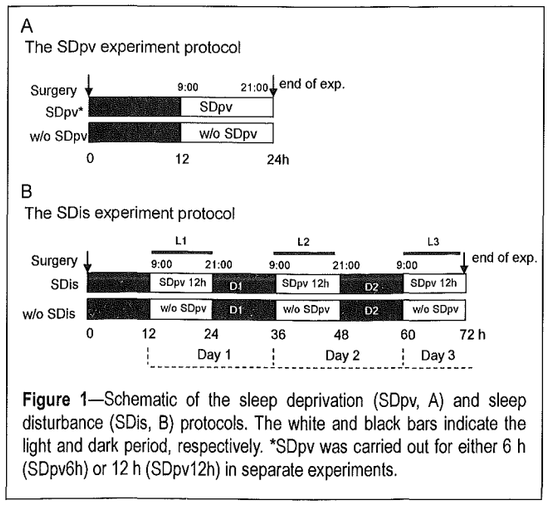


Figure 1—Schematic of the sleep deprivation (SDpv, A) and sleep disturbance (SDis, B) protocols. The white and black bars indicate the light and dark period, respectively. *SDpv was carried out for either 6 h (SDpv6h) or 12 h (SDpv12h) in separate experiments.

Plasma Corticosterone Determination

The plasma corticosterone level was measured in SDpv12h and SDis experiments. Blood samples were collected from trunk blood at the time when rats were decapitated at the end of SDpv (20:30-21:00, at the end of the light phase, Figure 1) and centrifuged with 2500 xg for 10 min. The plasma corticosterone concentration was later determined with Radio Immuno Assay kit (MP Biomedicals, Orangeburg, NY, USA), according to the manufacture's instruction.

Brain Damage Analysis

For each brain, 20 µm sections at 6 brain levels [A = +2.7 (L1), +1.7 (L2), +0.7 (L3), −0.3 (L4), −1.3 (L5), −2.3 (L6) from the bregma[22] were cut on a cryostat. Twelve to 15 sections at the each level were mounted on SuperFrost Plus slides (Menzel GmbH, Braunschweig, Germany) for histology assessments and remaining sections in each level were collected in RNase-free tubes for gene expression assay (see below). For assessing the infarct volume, sections were first fixed with phosphate-buffered saline (PBS, pH 7.4) containing 4% paraformaldehyde for 20 min at room temperature and then performed for standard cresyl violet staining. For assessing damaged cells, sections fixed with 4% paraformaldehyde were processed with terminal transferase biotinylated-dUTP nick end labeling (TUNEL) according to the manufacturer's instruction (Roche, Basel, Switzerland) to ascertain cells that contain fragmented DNA. On digitized cresyl violet sections, the infarct area were delineated and measured with the NIH imageJ software (NIH, Bethesda, MD, USA), and the infarct volume was converted with the known distance between each of the chosen levels and eventually corrected for edema by multiplying the ratio of the contralateral to ipsilateral volume.[23] Brain swelling was calculated with the formula as follows: [(ipsilateral − contralateral hemisphere)/contralateral hemisphere] × 100. The number of TUNEL positive cells was counted in the injured area measuring 20 mm^2 on sections collected at the level 6.

Taqman Gene Expression Assay

Brain tissues collected in RNase-free tubes (see above) were subjected to RNA extraction by the Trizol method (Life Technologies, Rockville, MD, USA) for individual animals. The total RNA was treated with RQ1 DNase (Promega, Madison, WI, USA) to digest genomic DNA. Oligo$(dT)_{15}$ primed the first-strand cDNA synthesis was synthesized by AMV reverse transcriptase (Promega). Group cDNA samples were synthesized by pooling together the RNA isolated from each animal. When necessary, cDNA from each rat was also synthesized to check the variation within group. The 5'-FAM labeled probes used in the Taqman real-time quantitative RT-PCR assay for GAPDH (endogenous control, Assay ID: Rn99999916_s1), GAP43 (Assay ID: Rn00567901_m1), c-JUN (Assay ID: Rn00572991_s1), neurocan (Assay ID: Rn00581331_m1), ephrinA5 (Assay ID: Rn005588118_s1), ephrinB1 (Assay ID: Rn00438666_s1) and glial fibrillary acidic protein (GFAP, Assay ID: Rn00566603_m1) were purchased from Applied Biosystems (Forster City, CA, USA). Reactions were performed in triplicates on AB 7900HT fast real time PCR system (Applied Biosystems). The relative level of mRNA expression in a given hemisphere was calculated as follows:

$$mRNA = 2^{-(\Delta C\,T\ \text{experiment group} - \Delta CT\ \text{sham w/o SDis})},$$

where $\Delta C_T = (C_{T,\,target} - C_{T,\,Gapdh})$

Immunoperoxidase Staining

Cryosections were fixed first with 4% paraformaldehyde in PBS for 20 min at room temperature and incubated overnight at 4°C with the primary antibodies against GFAP (raised in the rabbit, Dako, Glostrup, Denmark) and neurocan (raised in the mouse, Millipore, Billerica, MA, USA) diluted in PBS containing 2% normal goat serum (Jackson ImmunoResearch, West Grove, PA, USA) and 0.03% Triton X-100 (Sigma Chemicals, St. Louis, MO, USA). The concentration for the GFAP antibody was 1:6000; for the neurocan, it was 1:2000. Sections were washed with PBS, incubated with the biotin-conjugated secondary antibody (Jackson ImmunoResearch), diluted at 1:300 for 1 hour at room temperature, and processed with the ABC immunoperoxidase staining method using Vectastain Elite kits (Vector Laboratories, Burlingame, CA, USA) and diaminobenzidine (Sigma) as the chromogen. Stained sections were analyzed and photographed with Leica DM 6000B microscope (Leica Microsystems, Wetzlar, Germany).

Statistics

Data were presented as mean ± standard deviation (s.d.). The significance of differences in means was assessed by independent *t*-test, one-way analysis of variance (ANOVA), and repeated-measures ANOVA (SPSS, 12.01 for Windows) where appropriate, ANOVA was followed by post hoc comparisons to determine group differences. The significance level was set at P values < 0.05.

RESULTS

Neurological Score and Changes in Vigilance States

All animals subjected to MCAo did not exhibited neurological deficits according to Bederson's score,[21] i.e., without forelimb flexion when lifted up by the tail and without cycling behavior, both at 12 h after stroke and the end of experiments. They walked normally but were somewhat drowsier than sham operated rats.

Changes in the amount of EEG-defined vigilance states and in the episode length under the SDis procedure are listed in Table 1 and 2, respectively. SDpv12h carried out during the light phase resulted in an increase in SWS and PS during the following dark phase in both the ischemia/SDis and sham/SDis groups. In order to evaluate how SDpv12h influenced the total amount of sleep on a daily base in the SDis experiment, values in the light phases were combined with those in the following dark phase (total in Table 1). The SWS amount in the ischemia/SDis group reached almost the baseline level during day 1 and even tended to overshoot, although not statistically significant, during day 2, whereas in the sham group it was continuously below the baseline level by 11% to 12% (or 1.6 h) for 2 days. The difference between the 2 SDis-treated groups, i.e., ischemia/SDis 39.7 ± 3.7 vs. sham/SDis 28.2 ± 2.0 during day 1 and ischemia/SDis 47.9 ± 3.9 vs. sham/SDis 27.0 ± 1.6 during

day 2 (Table 1), was statistically significant (repeated-measures ANOVA, $P < 0.001$; independent *t*-test, $P < 0.001$), indicating an increased sleep propensity after ischemia. The total percentage of PS in the ischemia/SDis group decreased significantly from 10% of the baseline level (or 1.6 h) to 5% (or 0.8 h) during day 1 and recovered slightly during day 2. In the sham/SDis group PS decreased slightly (~0.5 h) for 2 days. As an effect of sleep deprivation, the episode length for SWS and PS was reduced during the light phase (L1, L2, and L3) but increased during the following dark phase for SWS (D1) (Table 2).

Delta power during SWS, a parameter for sleep pressure under normal conditions, increased in the sham/SDis group at the first 2 h following SDpv (Figure 2). In contrast, there was no change in the ischemia/SDis group (Figure 2). The difference between the two SDis groups suggests that the delta activity be altered after brain injury. When data were averaged for 8 h in the light or dark phase, there were no significant changes for all groups (not shown).

Effects of SDpv and SDis on Brain Damage

Occlusion of the distal MCA resulted in an infarct located in the primary somatosensory cortex. While SDpv6h barely ($P = 0.9$) influenced the infarct volume, SDpv12h increased it by 40% (SDpv12h 82.8 ± 10.9 vs. control 59.2+13.9 mm^3, $P = 0.008$) and SDis by 76% (SDis 58.8 ± 20.4 vs. control 33.8 ± 6.3 mm^3, $P = 0.017$), respectively (Figure 3A). The increased infarct area was found mostly at the level 5 and 6 (Figure 4), where a less arterial anastomosis may account for the vulnerability. In parallel to the change in the infarct size, SDpv12h increased the number of damaged cells, visualized by the TUNEL positive staining, by 137% (SDpv12h 46.8 ± 15 vs. control 19.7 ± 7.7/mm^2, $P = 0.003$) and SDis by 219% (SDis 32.9 ± 13.2 vs. control 10.3 ± 2.5/mm^2, $P = 0.008$), respectively (Figure 3B). There was no significant change in brain swelling in all experiments (Figure 3C).

To investigate how the exacerbated brain damage was influenced by changes in vigilance states, correlation of the infarct volume with altered W, SWS and PS was computed (Table 3). The results showed that the amount of W during day 1 was positively (Figure 5 and Table 3), and SWS negatively, correlated with the increased infarct size (Table 3). In addition, the episode length for SWS was also negatively correlated with the infarct size during three days of SDpv (L1 - L3) and for PS during the first two days (L1 and L2). These results suggest that both reduced and fragmented sleep contribute to the exacerbated stroke.

An additional experiment was carried out to determine whether sleep rebound following SDpv would alleviate the exacerbated brain damage induced by sleep disruption. In this experiment rats (n = 6) were subjected to the same procedure as these for SDpv12h but allowed to sleep for 24 h following SDpv before sacrificed. There was no difference ($P = 0.91$) in the infarct volume between this group (81.5 ± 24.7 mm^3) and the SDpv12h group (82.8 ± 10.9 mm^3), indicating that the SDpv12h-induced brain damage during the acute phase of stroke was not reversible.

Effects of SDis on Gene Expression

Changes in expression of neuroplasticity-related genes are summarized in Figure 6. The most striking change was the dramatic increase ($P < 0.001$) of the growth-inhibiting gene neurocan in the ischemic (ipsilateral) hemisphere in the ischemia/ SDis group (14.3 ± 0.4-fold), compared with the ischemia/ without SDis (6.1 ± 0.1-fold) and both sham groups (~1-fold). Additional assays with individual RNA samples were carried out to assess the variation within groups and the result confirmed the SDis-induced massive increase in neurocan expression ($P < 0.003$), with 11.03 ± 5.9-fold change in the ischemia/SDis group. SDis also induced a significant increase, although at smaller scale, in expression of another growth-inhibiting gene ephrinB1 in the injured hemisphere. In comparison, there was no SDis-induced further change in expression of GAP43, c-jun, and ephrinA5 (Figure 6A). In the contralateral hemisphere (Figure 6B), SDis slightly increased ($P < 0.05$) expression of GAP43, c-jun, neurocan, and ephrinB1, when compared with the ischemia/ without SDis.

Table 1—Changes in wakefulness (W), slow wave sleep (SWS), and paradoxical sleep (PS) in the SDis experiment

	Experiment groups (n = 6 for each group)											
	Ischemia w SDis			Ischemia w/o SDis			Sham w SDis			Sham w/o SDis		
Baseline	W§	SWS	PS§	W	SWS§	PS	W§	SWS§	PS§	W§	SWS	PS§
L	38.1 ± 6	51.8 ± 4	10.1 ± 4	42.3 ± 6	48.9 ± 6	8.8 ± 2	39.3 ± 5	51.6 ± 4	9.1 ± 3	38.7 ± 9	50.7 ± 8	10.6 ± 1
D	56.5 ± 4	33.5 ± 3	10.0 ± 4	59.6 ± 5	30.6 ± 3	9.8 ± 4	61.0 ± 6	27.7 ± 4	11.3 ± 2	69.4 ± 10	23.8 ± 9	6.8 ± 2
total	47.1 ± 4	42.9 ± 1	10.1 ± 3	50.9 ± 4	39.7 ± 4	9.3 ± 2	50.1 ± 4	39.6 ± 3	10.2 ± 2	54.1 ± 7	37.3 ± 7	8.7 ± 1
Day 1												
L	83.8 ± 6	15.8 ± 6	0.4 ± 1	39.2 ± 5	53.2 ± 5	7.6 ± 2	96.3 ± 1	3.7 ± 1	0.0 ± 0.0	33.5 ± 3	56.3 ± 4	10.1 ± 2
D	27.9 ± 8	61.9 ± 8	10.2 ± 4	49.6 ± 7	43.3 ± 7	7.1 ± 2	32.9 ± 4	52.3 ± 4	14.8 ± 2	53.9 ± 8	35.3 ± 6	10.8 ± 2
total	55.8 ± 4*	39.7 ± 3	5.2 ± 2*	44.8 ± 5	47.9 ± 3*	7.3 ± 2	64.6 ± 2*	28.2 ± 2*	7.4 ± 1*	42.2 ± 5*	46.3 ± 4	10.2 ± 1
Day 2												
L	77.0 ± 5	22.8 ± 5	0.2 ± 0.3	39.1 ± 10	54.1 ± 8	6.8 ± 3	93.6 ± 2	6.4 ± 2	0.0 ± 0.0	18.6 ± 7	52.9 ± 6	15.3 ± 2
D	30.8 ± 3	57.8 ± 10	11.4 ± 5	55.1 ± 9	37.0 ± 9	7.9 ± 4	37.9 ± 6	47.5 ± 4	14.6 ± 2	58.0 ± 9	32.4 ± 7	9.5 ± 2
total	53.2 ± 5*	47.9 ± 4	7.3 ± 2	47.5 ± 8	45.2 ± 5	7.4 ± 3	65.7 ± 2*	27.0 ± 2*	7.3 ± 1*	42.8 ± 7*	42.8 ± 7	12.3 ± 2*
Day 3												
L	85.1 ± 7	14.9 ± 7	0.0 ± 0	34.1 ± 9	59.8 ± 7	6.2 ± 3	94.0 ± 1	6.0 ± 1	0.0 ± 0.0	29.0 ± 2	57.4 ± 3	13.5 ± 2

Values are mean ± s.d. of percentages of recording hours (see Methods). L, the light phase (9:00-21:00, 8 h of recording time 11:00-19:00); D, the dark phase (21:00-9:00, 8 h of recording time 21:00-5:00); Total, both the light and dark phase combined (16h); §$P < 0.05$ in one-way ANOVA followed by Turkey post hoc comparison; *$P < 0.05$ when compared with the baseline of the corresponding phase.

Table 2—Changes in the episode length (s) of wakefulness (W), slow wave sleep (SWS), and paradoxical sleep (PS) in the SDis experiment

	Experiment groups (n = 6 for each group)											
	Ischemia w SDis			Ischemia w/o SDis			Sham w SDis			Sham w/o SDis		
L	W§	SWS§	PS§	W	SWS§	PS	W§	SWS§	PS§	W	SWS	PS
Baseline	128 ± 19	141 ± 33	80 ± 13	152 ± 44	119 ± 28	88 ± 20	143 ± 19	120 ± 16	69 ± 7	131 ± 21	125 ± 26	94 ± 15
L1	351* ± 82	69* ± 12	20* ± 22	182 ± 26	147 ± 24	93 ± 23	1044* ± 416	48* ± 3	0.0* ± 0	133 ± 12	144 ± 9	95 ± 16
L2	285* ± 93	96* ± 12	17* ± 29	196 ± 30	186* ± 44	101 ± 20	618* ± 150	47* ± 5	0.0* ± 0	122 ± 25	130 ± 31	110 ± 15
L3	378* ± 104	73* ± 11	0.0* ± 0	168 ± 40	215* ± 39	86 ± 27	604* ± 136	51* ± 6	0.0* ± 0	122 ± 12	155 ± 16	103 ± 16
D	W	SWS§	PS	W§	SWS§	PS	W§	SWS§	PS	W	SWS	PS
Baseline	347 ± 68	141 ± 17	104 ± 30	308 ± 39	114 ± 16	95 ± 24	414 ± 124	116 ± 12	116 ± 10	468 ± 162	120 ± 32	98 ± 17
D1	315 ± 174	297* ± 54	110 ± 17	320 ± 51	200* ± 40	108 ± 24	225* ± 36	206* ± 21	112 ± 12	337 ± 26	144 ± 21	114 ± 19
D2	287 ± 62	332 ± 110	111 ± 35	389* ± 55	167* ± 20	96 ± 33	239 ± 59	174* ± 19	113 ± 8	318 ± 72	120 ± 14	97 ± 10

Values are means (s) ± s.d. of the episode length (see Methods). L, the light phase (9:00-21:00, 8 h of recording time 11:00-19:00); D, the dark phase (21:00-9:00, 8 h of recording time 21:00-5:00); §$P < 0.05$ in One-way ANOVA followed by post hoc comparison; *$P < 0.05$ when compared with the baseline of the corresponding phase.

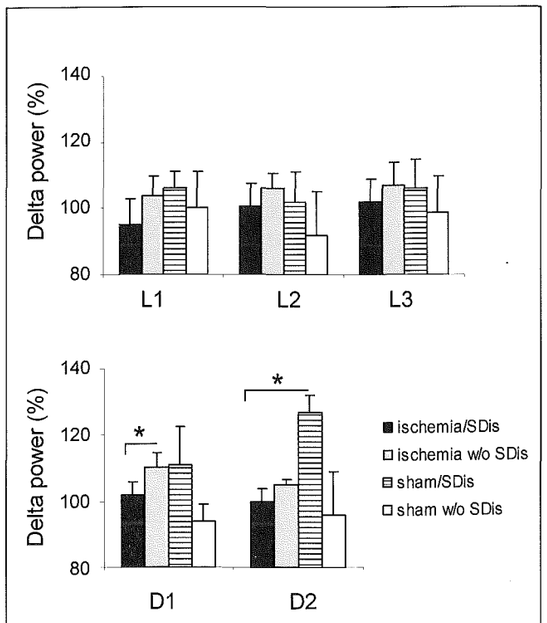


Figure 2—Effects of SDis on delta power (1-4 Hz) during slow wave sleep. Delta power was averaged for the first 2 h of each recording phase, and relative changes are plotted against the corresponding baseline (100%). Refer to Figure 1 for legends of L1, L2, L3, D1, and D2.

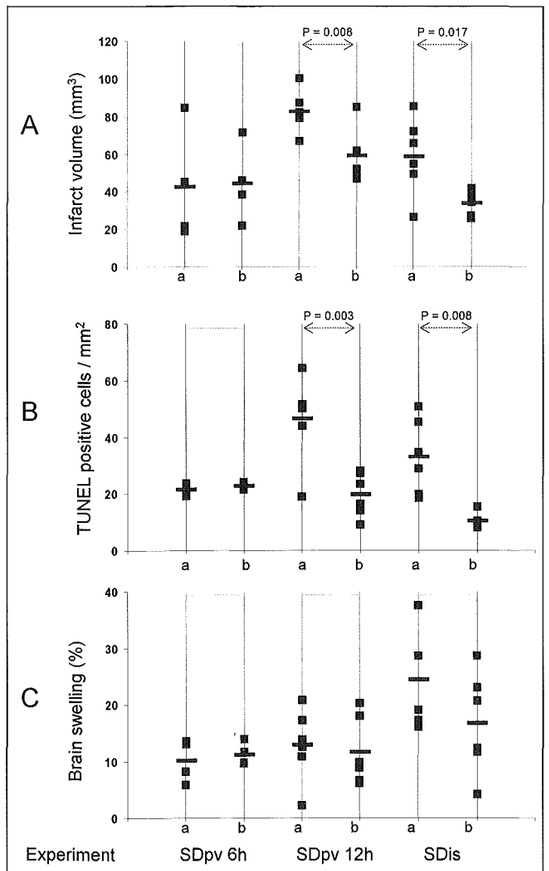


Figure 3—Scatter plots showing effects of SDpv and SDis on the infarct volume (A), the number of TUNEL positive cells (B), and brain swelling (C) in individual animals. Horizontal lines present the mean values and analyzed by independent *t*-test for each experiment. a, ischemia with sleep manipulation. b, ischemia without sleep manipulation.

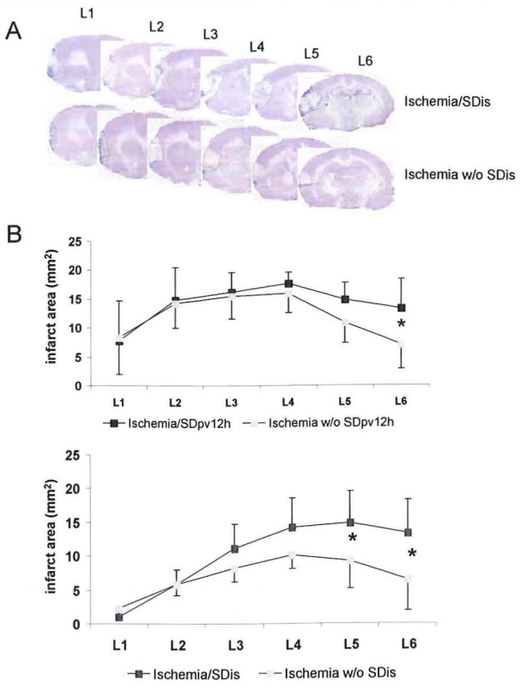


Figure 4—Effects of SDpv and SDis on the infarct area at different brain levels. A, Representative sets of brain sections from a rat subjected to ischemia/SDis (upper panel) and a rat to ischemia without (w/o) SDis (lower panel). The infarct areas are delineated by a black thin line. L1 is at 2.7 mm anterior to bregma, and the interval between each level is 1 mm (Methods). B, Values are presented as mean ± s.d. (n = 6 per group) and analyzed by independent *t*-test. *P < 0.05.

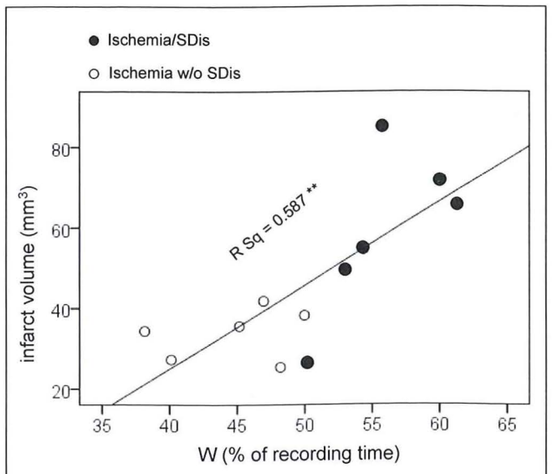


Figure 5—Correlation of the infarct volume with the amount of wakefulness (W) during the first day of the sleep disturbance. W values are the percentage of the total recording time including both the light and dark phase (Table 1, total).

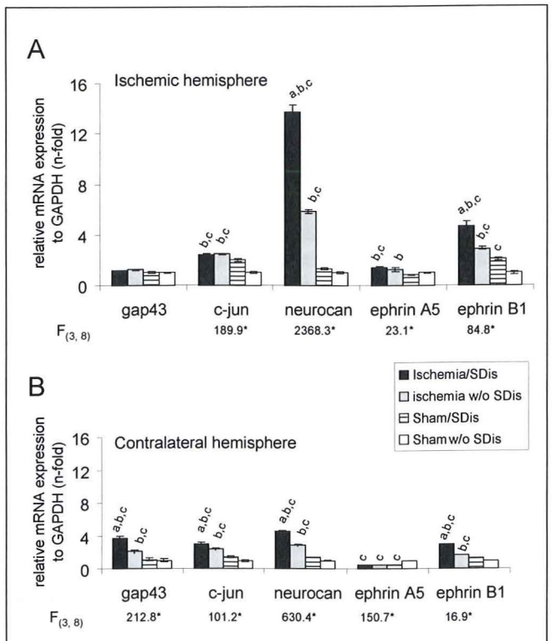


Figure 6—Effects of SDis on expression of neuroplasticity-related genes. Values are presented as mean ± s. d. ᵃP < 0.05 when compared with the ischemia w/o SDis group. ᵇP < 0.05 when compared with the sham w. SDis group. ᶜP < 0.05 when compared with the sham w/o SDis group. One-way ANOVA (see F values, *P < 0.001) followed by Turkey post hoc comparisons.

Table 3—Correlaton of infarct size with wakefulness (W), slow wave sleep (SWS), and paradoxical sleep (PS) in the SDis experiment.

Infarct (mm³)	Day	R Sq	Pearson	P
W (%)	1	0.587	0.766**	0.004
	2	0.201	0.449	0.193
SWS (%)	1	0.592	−0.770**	0.003
	2	0.246	−0.496	0.144
PS (%)	1	0.007	−0.082	0.8
	2	0.07	−0.264	0.461
W episode length (s)	L1	0.413	0.642*	0.024
	L2	0.513	0.716**	0.009
	L3	0.637	0.798**	0.002
SWS episode length (s)	L1	0.339	−0.582*	0.047
	L2	0.352	−0.593*	0.042
	L3	0.413	−0.642*	0.024
PS episode length (s)	L1	0.453	−0.673*	0.016
	L2	0.689	−0.830**	0.001
	L3	0.361	−0.601	0.039

The amount of each vigilance states is presented as percentage of total recording time for each day. The episode length values are from the sleep deprivation period and refer to Figure 1 for L1, L2, and L3. *P < 0.05; **P < 0.01.

Expression of GFAP and Localization of Neurocan

The growth-inhibiting gene neurocan has been known to be secreted by reactive astrocytes in response to various CNS injures,[24] including ischemic stroke.[25,26] To assess whether the SDis-induced massive increase in neurocan expression was parallel to an increase in reactive astrocytes, we determined the expression of the astrocyte marker GFAP with Taqman assay and the cellular distribution of both GFAP and neurocan with immunoperoxidase staining in the ischemic hemisphere. The GFAP mRNA level increased, although at much smaller scale (2-fold) compared to the increase in the expression of neurocan, in the ischemia without SDis group when compared with sham groups, and there was a slightly (but significantly) further increase in the ischemia/SDis (Figure 7A). The immunoreactivity for both GFAP and neurocan was highly expressed in the peri-infarct area (Figure 7B and C).

Plasma Corticosterone level after SDpv12h and SDis

In the SDpv12h experiment, there was no significant change in the plasma corticosterone level between the ischemia/SDpv12h and its control group. In the SDis experiment, there was a significant decrease in groups subjected to SDis (ischemia and sham) when compared with groups without SDis. There was no significant difference between the ischemia/SDis and sham/SDis (Figure 8).

Changes in Body Weight

Ischemia-operated rats tended to lose about 8-15 g or 5% of their body weight after surgery, compared to 0-4 g of sham-operated rats (Table 4). In neither the ischemia/SDpv12h nor the ischemia/SDis group did the body weight change significantly when compared with their paired ischemia/without SDpv12h or ischemia/without SDis group.

Table 4—Changes in body weight after SDpv12h and SDis

Experiment	Groups baseline weight (g)	Day 1*	Day 2*	Day 3*
SDpv12h (n = 6 per group)	Ischemia/SDpv 262 ± 31	−14.3 ± 6.1		
	Ischemia w/oSDpv 257 ± 23	−14.8 ± 6.1		
SDis§ (n = 6 per group)	Ischemia/SDis 272 ± 17	−12.2 ± 2.3ᵃ,ᵇ	−17.0 ± 6.1ᵃ,ᵇ	−11.7 ± 3.7ᵇ
	Ischemia w/o SDis 324 ± 25	−8.2 ± 5.6ᵇ	−10.5 ± 5.9ᵇ	−10.3 ± 6.4
	Sham/SDis 296 ± 24	−4.0 ± 5.2	−7.8 ± 6.4	−9.8 ± 6.4
	sham w/o SDis 305 ± 14	0.0 ± 5.2	−0.1 ± 5.3	1.3 ± 4.4

*Values are means of difference from the baseline ± standard deviation. §Repeated measures ANOVA ($F_{3, 20}$ = 5.56, P = 0.006) followed by independent *t*-test. ᵃP < 0.05 when compared with sham/SDis. ᵇP < 0.05 when compared with the sham w/o SDis.

DISCUSSION

The findings that both sleep deprivation and repeated sleep disruption over 3 days aggravate acute brain damage (Figure 3

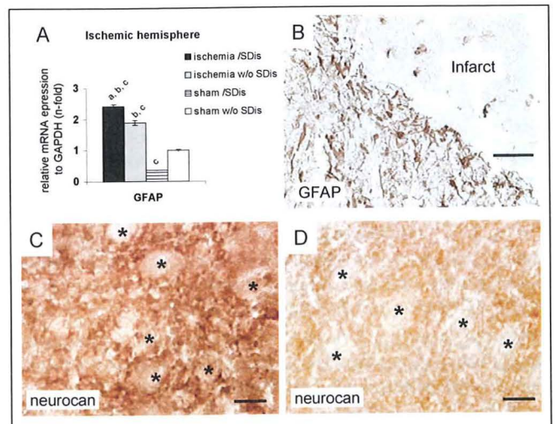


Figure 7—Expression of the GFAP gene (A) and distribution of the immunoreactivity (ir) for GFAP (B) and neurocan (C, D). A: Values are presented as mean ± s.d. One-way ANOVA ($F_{3, 8}$ = 1013. *P < 0.001) followed by Games-Howell comparisons. ᵃP < 0.05 when compared with the ischemia w/o SDis group. ᵇP < 0.05 when compared with the sham w. SDis group. ᶜP < 0.05 when compared with the sham w/o SDis group. B. The GFAP-ir in the peri-infarct area. Note the highly expressed GFAP-ir around the border of the infarct area. Bar, 100 μm. C and D, High-power photomicrograph illustrating expression of the neurocan-ir around parenchyma cells (marked with *). Note the substantially increased expression of the neurocan-ir in the peri-infarct area (C) compared with the weak staining in the contralateral cortex (D). Bars, 20 μm.

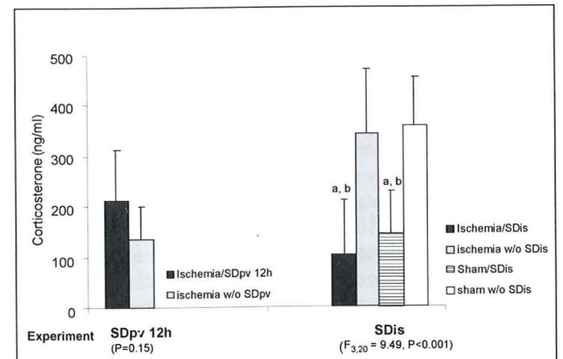


Figure 8—Effects of SDpv and SDis on the plasma corticosterone level. Values are presented mean ± s.d. (n = 6 per group) and analyzed by either independent *t*-test in the SDpv12h experiment or one-way ANOVA followed by Turkey post hoc comparisons in the SDis experiment. ᵃP < 0.02 when compared with the ischemia w/o SDis group. ᵇP < 0.02 when compared with the sham w/o SDis group.

and 4) provide the first direct evidence for a detrimental effect of sleep disruption on stroke. The underlying mechanism(s) remains, however, to be specified. It has been known that high levels of plasma glucocorticoids after ischemia increase neuronal vulnerability,[27] whereas low levels reduce brain damage.[28] In this study, SDpv12h did not significantly alter the corticosterone level (Figure 8), while SDpv12h for consecutive 3 days (the SDis experiment) decreased the level (Figure 8). Thus, the plasma corticosterone level does not appear to be related to the worsened brain damage after SDpv/SDis. Ischemic stroke, due to a sudden reduction of blood flow, triggers a series of events, including energy failure, excitotoxicity, free radical generation, and inflammation—a cascade that determines the fate of infarction.[4,5] Short-term SDpv has been shown to elevate the brain temperature[29] and brain energy consumption, evidenced by increasing 2-deoxyglucose uptake[8] and decreasing the level of brain glycogen, the principal energy store in the brain.[6] Short-term SDpv also increases the extracellular glutamate concentration,[9] glutamate receptor levels and neuronal activities,[30] which would worsen ischemia-induced excitotoxicity. Furthermore, prolonged SDpv has been shown to decrease levels of some antioxidative stress markers, such as glutathione, glutathione peroxidase, and superoxide dismutase,[31-33] which in turn could intensify cell damage caused by free oxygen radical in the ischemia site. Prolonged SDpv or sleep restriction also increases levels of certain proinflammatory cytokines such as IL-1ß, IL-6, and TNFα[10,11] that play an important role in the inflammatory phase of cerebral ischemia.[34] Taken together, substantial experimental evidence suggests that SDpv/SDis could aggravate stroke through potentiation of mechanisms implicated in its pathophysiology.[4,5] Further studies are needed to identify essential contributors.

SDis altered the expression of several genes that mediate initiation of post-stroke axonal sprouting at the early phase after stroke (Figure 6). The substantial changes occurred in the ischemic hemisphere where SDis induced a massive increase in expression of the growth-inhibiting gene neurocan (Figure 6). Neurocan is one of major chondroitin sulfate proteoglycans (CSPGs) and secreted by reactive astrocytes in response to various CNS injures.[24-26] Its inhibitory property to axonal growth has been demonstrated both in vitro[35,36] and in vivo,[26,37] possibly by activation of the Rho/ROCK pathway.[38] Around the border of the infarct area, densely packed reactive astrocytes (Figure 7B) together with growth-inhibitory molecules including neurocan (Figure 7C) form a physical and biochemical barrier that hinders the neuronal reconnection. Interestingly, the present data that SDis increases neurocan expression after ischemic stroke is in line with our previous observations that the sleep stimulant gamma-hydroxybutyrate has an opposite (decrease) effect on the ischemia-induced neurocan expression,[16] suggesting that neurocan be an important molecule implicated in the sleep-modulated post-stroke brain plasticity. In this context, large scale screening of neuroplasticity-related genes and their protein products is needed to understand molecule mechanisms involved in sleep-modulated neuroplasticity after ischemic stroke. In addition, it remains to demonstrate the influence of sleep disruption on post-stroke axonal sprouting and finally on functional recovery.

In summary, this study demonstrate that at the early phase of stroke, sleep disruption aggravates brain damage and increases expression of genes that inhibit post-stroke axonal sprouting. Thus, prevention of sleep disturbances and improvement of sleep quality may become a new therapeutic window to improve outcome.

ACKNOWLEDGMENTS

The authors thank Dr. Jyrki Eloranta, Division of Clinical Pharmacology and Toxicology, University Hospital Zürich, for his help with the Taqman assay.

The work was performed at University Hospital Zürich, Switzerland.

DISCLOSURE STATEMENT

This was not an industry supported study. The authors have indicated no financial conflicts of interest.

REFERENCES

1. Bassetti CL. Sleep and stroke. Semin Neurol 2005;25:19-32.
2. Hermann DM, Siccoli M, Brugger P, et al. Evolution of neurological, neuropsychological and sleep-wake disturbances after paramedian thalamic stroke. Stroke 2008;39:62-8.
3. Siccoli MM, Rolli-Baumeler N, Achermann P, Bassetti CL. Correlation between sleep and cognitive functions after hemispheric ischaemic stroke. Eur J Neurol 2008;15:565-72.
4. Dirnagl U, Iadecola C, Moskowitz MA. Pathobiology of ischaemic stroke: an integrated view. Trends Neurosci 1999;22:391-7.
5. Durukan A, Tatlisumak T. Acute ischemic stroke: overview of major experimental rodent models, pathophysiology, and therapy of focal cerebral ischemia. Pharmacol Biochem Behav 2007;87:179-97.
6. Kong J, Shepel PN, Holden CP, Mackiewicz M, Pack AI, Geiger JD. Brain glycogen decreases with increased periods of wakefulness: implications for homeostatic drive to sleep. J Neurosci 2002;22:5581-7.
7. Stickgold R. Sleep-dependent memory consolidation. Nature 2005;437:1272-8.
8. Vyazovskiy VV, Cirelli C, Tononi G, Tobler I. Cortical metabolic rates as measured by 2-deoxyglucose-uptake are increased after waking and decreased after sleep in mice. Brain Res Bull 2008;75:591-7.
9. Dash MB, Douglas CL, Vyazovskiy VV, Cirelli C, Tononi G. Long-term homeostasis of extracellular glutamate in the rat cerebral cortex across sleep and waking states. J Neurosci 2009;29:620-9.
10. Shearer WT, Reuben JM, Mullington JM, et al. Soluble TNF-alpha receptor 1 and IL-6 plasma levels in humans subjected to the sleep deprivation model of spaceflight. J Allergy Clin Immunol 2001;107:165-70.
11. Vgontzas AN, Zoumakis E, Bixler EO, et al. Adverse effects of modest sleep restriction on sleepiness, performance, and inflammatory cytokines. J Clin Endocrinol Metab 2004;89:2119-26.
12. Nudo RJ, Wise BM, SiFuentes F, Milliken GW. Neural substrates for the effects of rehabilitative training on motor recovery after ischemic infarct. Science 1996;272:1791-4.
13. Traversa R, Cicinelli P, Bassi A, Rossini PM, Bernardi G. Mapping of motor cortical reorganization after stroke. A brain stimulation study with focal magnetic pulses. Stroke 1997;28:110-7.
14. Carmichael ST, Archibeque I, Luke L, Nolan T, Momiy J, Li S. Growth-associated gene expression after stroke: evidence for a growth-promoting region in peri-infarct cortex. Exp Neurol 2005;193:291-311.
15. Carmichael ST, Chesselet MF. Synchronous neuronal activity is a signal for axonal sprouting after cortical lesions in the adult. J Neurosci 2002;22:6062-70.
16. Gao B, Kilic E, Baumann CR, Hermann DM, Bassetti CL. Gamma-hydroxybutyrate accelerates functional recovery after focal cerebral ischemia. Cerebrovasc Dis 2008;26:413-9.
17. Cirelli C, Gutierrez CM, Tononi G. Extensive and divergent effects of sleep and wakefulness on brain gene expression. Neuron 2004;41:35-43.
18. Cirelli C, Faraguna U, Tononi G. Changes in brain gene expression after long-term sleep deprivation. J Neurochem 2006;98:1632-45.
19. Trachsel L, Tobler I, Borbely AA. Electroencephalogram analysis of non-rapid eye movement sleep in rats. Am J Physiol 1988;255:R27-37.
20. Tamura A, Graham DI, McCulloch J, Teasdale GM. Focal cerebral ischaemia in the rat: 1. Description of technique and early neuropathological consequences following middle cerebral artery occlusion. J Cereb Blood Flow Metab 1981;1:53-60.
21. Bederson JB, Pitts LH, Tsuji M, Nishimura MC, Davis RL, Bartkowski H. Rat middle cerebral artery occlusion: evaluation of the model and development of a neurologic examination. Stroke 1986;17:472-6.
22. Paxinos G, Watson C. The rat brain in stereotaxic coordinates. Sixth edition. London, Amsterdam, Burlington: Elsevier 2007.
23. Junge CE, Sugawara T, Mannaioni G, et al. The contribution of protease-activated receptor 1 to neuronal damage caused by transient focal cerebral ischemia. Proc Natl Acad Sci U S A 2003;100:13019-24.
24. Sandvig A, Berry M, Barrett LB, Butt A, Logan A. Myelin-, reactive glia-, and scar-derived CNS axon growth inhibitors: expression, receptor signaling, and correlation with axon regeneration. Glia 2004;46:225-51.
25. Deguchi K, Takaishi M, Hayashi T, et al. Expression of neurocan after transient middle cerebral artery occlusion in adult rat brain. Brain Res 2005;1037:194-9.
26. Shen LH, Li Y, Gao Q, Savant-Bhonsale S, Chopp M. Down-regulation of neurocan expression in reactive astrocytes promotes axonal regeneration and facilitates the neurorestorative effects of bone marrow stromal cells in the ischemic rat brain. Glia 2008;56:1747-54.
27. Sapolsky RM, Pulsinelli WA. Glucocorticoids potentiate ischemic injury to neurons: therapeutic implications. Science 1985;229:1397-400.
28. Krugers HJ, Knollema S, Kemper RH, Ter Horst GJ, Korf J. Down-regulation of the hypothalamo-pituitary-adrenal axis reduces brain damage and number of seizures following hypoxia/ischaemia in rats. Brain Res 1995;690:41-7.
29. Franken P, Dijk DJ, Tobler I, Borbely AA. Sleep deprivation in rats: effects on EEG power spectra, vigilance states, and cortical temperature. Am J Physiol 1991;261:R198-208.
30. Vyazovskiy VV, Cirelli C, Pfister-Genskow M, Faraguna U, Tononi G. Molecular and electrophysiological evidence for net synaptic potentiation in wake and depression in sleep. Nat Neurosci 2008;11:200-8.
31. D'Almeida V, Lobo LL, Hipolide DC, de Oliveira AC, Nobrega JN, Tufik S. Sleep deprivation induces brain region-specific decreases in glutathione levels. Neuroreport 1998;9:2853-6.
32. Ramanathan L, Gulyani S, Nienhuis R, Siegel JM. Sleep deprivation decreases superoxide dismutase activity in rat hippocampus and brainstem. Neuroreport 2002;13:1387-90.
33. Singh R, Kiloung J, Singh S, Sharma D. Effect of paradoxical sleep deprivation on oxidative stress parameters in brain regions of adult and old rats. Biogerontology 2008;9:153-62.
34. Mergenthaler P, Dirnagl U, Meisel A. Pathophysiology of stroke: lessons from animal models. Metab Brain Dis 2004;19:151-67.
35. Friedlander DR, Milev P, Karthikeyan L, Margolis RK, Margolis RU, Grumet M. The neuronal chondroitin sulfate proteoglycan neurocan binds to the neural cell adhesion molecules Ng-CAM/L1/NILE and N-CAM, and inhibits neuronal adhesion and neurite outgrowth. J Cell Biol 1994;125:669-80.
36. Asher RA, Morgenstern DA, Fidler PS, et al. Neurocan is upregulated in injured brain and in cytokine-treated astrocytes. J Neurosci 2000;20:2427-38.
37. Davies JE, Tang X, Denning JW, Archibald SJ, Davies SJ. Decorin suppresses neurocan, brevican, phosphacan and NG2 expression and promotes axon growth across adult rat spinal cord injuries. Eur J Neurosci 2004;19:1226-42.
38. Monnier PP, Sierra A, Schwab JM, Henke-Fahle S, Mueller BK. The Rho/ROCK pathway mediates neurite growth-inhibitory activity associated with the chondroitin sulfate proteoglycans of the CNS glial scar. Mol Cell Neurosci 2003;22:319-30.