

# Physiological Research Pre-Press Article

## **Impacts of perinatal induced photothrombotic stroke on sensorimotor performance in adult rats.**

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## **Impacts of perinatal induced stroke tested in adult rats.**

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## **Abstract**

Perinatal ischemic stroke is a leading cerebrovascular disorder occurring in infants around the time of birth associated with long term comorbidities including motor, cognitive and behavioral deficits. We sought to determine the impact of perinatal induced stroke on locomotion, behavior and motor function in rats.

A photothrombotic model of ischemic stroke was used in rat at postnatal day 7. Presently, we induced two lesions of different extents, to assess the consequences of stroke on motor function, locomotion and possible correlations to morphological changes. Behavioral tests sensitive to sensorimotor changes were used; locomotion expressed as distance moved in the open field was monitored and histological changes were also assessed.

Outcomes depicted two kinds of lesions of different shapes and sizes, relative to laser illumination. Motor performance of rats submitted to stroke was poor when compared to controls; a difference in motor performance was also noted between rats with small and large lesions. Correlations were observed between: motor performance and exposition time; volume ratio and exposition time; and in the rotarod between motor performance and volume ratio. Outcomes demonstrate that photothrombotic cerebral ischemic stroke induced in early postnatal period and tested in adulthood, indeed influenced functional performance governed by the affected brain regions.

**Keywords:** photothrombosis; perinatal ischemic stroke; sensorimotor performance; immature rats

## **Introduction**

Perinatal ischemic stroke is the leading cerebrovascular disorder occurring in infants around the time of birth with pathological evidence of focal arterial infarction (de Vries et al. 1997; Nelson and Lynch 2004). Symptomatic perinatal ischemic stroke is experienced by about one in every 4000 term births (Estan and Hope 1997; Lynch and Nelson 2001) and is associated with high medical and social costs, due to long-term co-morbidity (Sran and Baumann 1988; Sreenan et al. 2000). Despite a lack of symptoms during early postnatal periods, studies has shown that the numbers of reported neonatal stroke cases are on the increase in some areas (Edwards & Azzopardi 2000; Miller et al. 2004; Wu et al. 2005) thus the actual incidence is likely higher than the above written value. Up to 27% of infants with these brain injuries later in life develop numerous consequent pathologies as well as behavioral and functional deficits. Among these are speech delays, hemi-paresis and hemi-sensory impairments (Westmacott et al. 2009; Vinay et al. 2005; Hattori et al. 2000). Clinically, little is known concerning long-term behavioral outcome after neonatal stroke (Westmacott et al. 2009), this fact holds true also in experimental studies. There is a shortage of stroke models focused purely on long-term neurobehavioral and functional outcomes induced at an immature age and assessed in adulthood. Existing work of this nature documented a correlation between the degree of brain injury in neonatal rats and functional deficits such as asymmetries of limb placing, foot-faults and abnormality in the postural reflex tests (Bona et al. 1997). Although neonatal stroke is increasingly being studied, most of the foundation of our understanding on functional and behavioral endpoints comes from research done in adult animal models. In order to study perinatal stroke, several methods to induce ischemic brain damage have been elaborated in immature rats at postnatal day (P) 7 (Ashwal et al. 1995; Renolleau, et al. 1998; Rice III et al. 1981). Research evinced that rat's brain at this

age is as mature as the brain of a human fetus in the third trimester; with regard to parameters such as number of synapses, neurochemical development and cortical organization (Hagberg et al. 1997); making it the appropriate age for this study. Hypoxic ischemic (HI) based methods are dominating in this field, but prove to be technically challenging to perform at an early age resulting in inconsistent infarct volume and high mortality rates. In contrast, photothrombosis is a less invasive method that can be performed at an immature age (Maxwell and Dyck 2005). This method is based on a thrombus-producing photochemical reaction of photosensitive dyes activated by green laser light that typically produces a consistent infarct of a specific location and size (Watson and Padro 2009; Karhunen et al. 2007; Matějovská et al. 2008).

In the present study we used the Bengal Rose model of photothrombotic stroke to induce two ischemic lesions of different extends, at postnatal day (P) 7. This was done in order to assess consequences of perinatal stroke on motor performance, behavioral and functional responses, such as posture and motor coordination in adulthood. To this end, animals were submitted to four simple behavioral tests (rotarod, bar holding, inclined grid, and ladder rung walking) sensitive to sensorimotor changes caused by various brain injuries. Moreover, locomotor activity expressed as distance moved in the open field (OF) was monitored.

## **Experimental Procedure**

### *Animals*

Experiments were performed in male albino Wistar rats (n = 139), provided by the Institute of Physiology of the Academy of Sciences of Czech Republic. At postnatal day (P) 7 the animals were brought to the experimental room and divided into four groups: animals (n=30) exposed to laser light for 5 min. (BR\_5 min); animals (n=38) exposed to laser light for 30 sec. (BR\_30 s);

sham-operated controls (Sham) treated with saline (n=31) and intact control group (C, n=40). Behavioral tests were performed at P 67. A group of seventy-eight rats (BR\_5 min = 20, BR-30 s = 19, Sham = 19 and C = 20) were submitted to rotarod, bar holding and inclined grid tests. A second group of sixty-one rats (BR\_5 min = 10, BR\_30 s = 19, Sham = 12 and C = 20), were submitted to ladder rung walking tests and open field (OF). A subset of twenty-one animals, n = 7 from each experimental group, were used to confirm the ischemic injury. Rats were housed in standard plastic cages in a temperature-controlled environment of 22±1 °C, humidity 50-60% with a 12-h light/dark cycle (lights on at 6 a.m.) with free access to food and water. All efforts were made to minimize animal suffering and to reduce the number of animals used.

The protocol of the experiments was approved by the animal Care and Use Committee of the Institute of Physiology, Academy of Sciences of the Czech Republic (v.v.i) to be in agreement with the Animal Protection Law of the Czech Republic, which is fully compatible with the guidelines of the European Community Council directives 86/609/EEC.

## **Surgical procedures**

### *Induction of Photothrombosis*

Cortical brain lesions were induced by photothrombosis according to Watson and Padro (2009) with slight modifications. Before the procedures started rats were weighted and marked. They were anesthetized with isoflurane (1.5 – 5%). A midline scalp incision was made and the scalp was retracted laterally to expose the skull. Freshly prepared Bengal Rose solution (Sigma®, BR 10 mg/1 ml, 0.15 ml per animal) was injected into the exposed right jugular vein, rats were then transferred within 1 minute to a stereotaxic frame and their brains were exposed (through an intact skull) to Green laser light (Roithner Laser, Austria 50 mW output) for 60 sec at 0.5s on/off

cycles. The center of the light beam was focused on the left sensorimotor cortex, 1.8 mm from Bregma anterior posteriorly (AP) and 2.8 mm laterally (L) extending to the midline medially AP = 1.8mm, L = 2.8mm (Paxinos and Watson 1998). The incisions were immediately sutured and collodium was applied on the suture. Animals were allowed to recover from anesthesia for about 45 minutes before returning to their mother. Sham-operated controls followed the same protocol, except that saline was used instead of Bengal Rose injection before laser light illumination. Two animals were removed from experiment due to procedural problems during surgery. Body temperatures were monitored and maintained at 37° C throughout the surgery. The health conditions of animals were checked regularly.

### **Behavioral testing**

Behavioral testing was performed in adult animals, 60 days after photothrombosis. The deficits of weight supported stepping, paws coordination, endurance, muscle strength, skilled walking, were assessed with a battery of simple tests adopted from our previous work (Mikulecká et al. 2002; Ticha et al. 2011). Before behavioral testing, all animals were weighted and left to adapt to the experimental room for one hour.

#### *Rotarod test*

Motor coordination and equilibrium was observed using an automatic rotarod treadmill unit, set to rotate at a constant 5rpms and placed 30 cm above a landing platform. The unit consisted of a horizontal rod (10cm in diameter 11cm long) covered with thick plaster to increase its roughness. The rats were placed individually on the rotating rod with their heads directed against the direction of rotation. Three trials were performed in a close succession. Maximal score in maintaining equilibrium was arbitrarily fixed at 60 seconds. The mean of three trials was taken

as a score. A video camera was placed in front of the rotarod, to observe the behavioral strategies used by an animal to maintain equilibrium. Two strategies were observed passive rotation and walking. The passive rotation consisted in hanging on the rotarod and being passively rotated. During the walking behavior the rat walked asynchronously (the movement of the paws was not perfectly coordinated) and synchronously (the rat walked evenly, the paws being perfectly coordinated).

#### *Bar holding*

An animal was held by the nape and its forepaws were allowed to touch a wooden bar (25 cm long, 1 cm in diameter, suspended 25 cm above a padded soft surface). Time of fore- and hind-limb grasping was recorded with a limit of 60 sec. The mean of the three grasping latencies was taken as the score.

#### *The inclined grid*

Turning on the inclined grid was assessed using a modification of the procedure described by Marshall (1982). A grid (75 cm with x 100 cm height) made of stainless was held in a horizontal position. A rat was placed on it, approximately in the center. Then, the grid was lowered until it attained a negative inclination of 30 with respect to the horizontal plane. The latency to turn on the grid to 180° was measured for a maximum of 60 sec. If the rat failed to turn on the grid, the latency was set to 60 sec. The mean of three turning latencies was taken as a score.

#### *The ladder rung walking*

The ladder rung walking test is used to assess motor and locomotor performances specifically skilled walking, forelimb and hind limb placing, stepping, and inter-limb co-ordination and

balance (Metz and Whishaw 2002). In the present study we used this test to assess the ability of rats to successfully cross the runway. A horizontal ladder walking apparatus that consisted of side walls made of clear Plexiglas and metal rungs (3 mm diameter), that could be inserted to create a floor with a minimum distance of 1cm between rungs was used. The ladder was elevated 30 cm above the ground with a empty starting cage and a refuge (home cage) at the end. The width of the alley was adjusted to the size of the animal, to prevent the animal from turning around. The time to cross the runway in one trial with regular gaps and another trial with irregular gaps was assessed. The rats were tested with a limit of 60 sec.

### *Open field*

The open field (OF) test was performed in a square arena (45x45x30 cm), with a camera installed above the center. Immediately after a rat was placed in the center of the arena; locomotor behavior was recorded automatically by a computerised system (Etho Vision Noldus Information Technology) for 5 min. Locomotor activity expressed as distance moved (cm) was calculated.

To reduce any lingering olfactory cues all devices were cleaned after each rat was tested.

### **Histology**

One week after behavioral testing, animals were overdosed with urethane (2g/kg i.p.), and perfused transcardially with ice cold 0.01 M phosphate-buffered saline (PBS; pH 7.5) followed by 4% formaldehyde. The brains were removed and post fixed in 4% formaldehyde at 4°C and then sequentially soaked in 10%, 20% and 30% sucrose. The brains were then frozen in dry ice and stored at -70°C. Coronal sections (50µm thick; Cryocut Leika 1600) were cut through the entire brain and every fifth section was collected and placed in 4% formaldehyde. To assess cell



morphology, brain sections were derived from all investigated rats, mounted onto gelatin-coated slides and stained with cresyl violet or Nissl staining (Otahal et al. 2005). Slides were cover-slipped and assessed using a light microscope (Olympus® AX 70 microscope with bright field optics).

### *Evaluation*

To evaluate the magnitude of lesions; ratios between hemispheres (affected/contralateral) were calculated. Volumes of these hemispheres were stereologically estimated by Cavalieri principle and point-grid probe in the Ellipse software (ViDiTo, Slovakia). Briefly, every fifth slice (50µm thick) was obtained from a cutting series of slices throughout the brain. These slices were stained with Nissl and placed under a stereomicroscope, to capture their images, with a digital camera. A set of slices from one brain and a measuring scale bar were captured using the same magnification and camera setting. The obtained images included both hemispheres; this allowed us to use the same settings in the point-grid for both hemispheres, affected and non-affected contralateral hemispheres. Spacing of the point-grid probe was set to 0.2mm.

### **Statistics**

Statistical analysis was performed with (Sigma Stat3.5®SPSS) program. To assess the difference in tissue volume, non parametrical Mann-Whitney test was used. Pearson Product Moment Correlation was used to detect correlations between motor performance and volume ratio and between volume ratio and duration of laser light exposure. Sensorimotor performance data were analysed using one-way ANOVA with post hoc Student-Newman-Keuls Method. The differences in the ratio of correct response (animals crossed the ladder rung walking within the

60 sec.) were evaluated by means of Chi<sup>2</sup> -test. All data was expressed as mean ± SEM and statistical significance  $P < 0.05$  was accepted.

## **Results**

### *Morphology of the photothrombotic lesions*

Two different exposition times to green laser light (30 s and 5 min) focused on sensorimotor cortex, responsible for sensorimotor limb function (Castro-Alamancos and Borrel 1995), were chosen to induce focal cerebral ischemia in 7 days old rats. The shorter exposition time (30 s - BR\_30 s) induced lesions which maturates to diameters ranging from 0.3 – 2.5mm when assessed in adult animals, penetrating through all cortical layers leaving subcortical white matter intact (Fig.1A). The longer exposition time (5 min - BR\_5 min) induced larger lesions of 2.4 - 3.7 mm in diameter involving all cortical layers including the underlying white matter and subcortical structures (e.g. striatum) of affected hemisphere (Fig.1B). Volume ratios between hemispheres (affected/contralateral) were calculated; the animals with large lesion presented a mean volume ratio of  $(0.79 \pm 0.07)$  ischemic hemisphere versus the contralateral hemisphere, while those with small lesion had a mean volume ratio of  $(0.94 \pm 0.02)$ . Mann-Whitney test revealed a significant difference in volume ratio between rats with longer exposition time and controls, intact (C) and sham-operated (Sham); also between both controls (C and Sham) and rats with shorter exposition time (Fig.1C). Pearson Product Moment Correlation also revealed significant negative correlations between volume ratio and exposition time. All control animals, intact and sham-operated, presented no structural evidence of cerebral damage.

### *Behavioral testing*

The results of behavioral testing are summarized in Fig. 2

#### *Rotarod test*

The ability to coordinate and balance on a rotating drum was tested in adult rats, 60 days after photothrombosis. The overall analysis of the time spent on the rotarod revealed significant main effects of experimental condition ( $F(3, 74) = 24.62, p < 0.001$ ). Both groups of rats with either small lesions or large lesions walked for a significantly shorter time on the rotarod ( $29.16 \pm 3.79$  s and  $20.23 \pm 2.5$  s, respectively) compared to controls (C)  $51.57 \pm 2.22$  s and Sham-operated rats  $45.6 \pm 3.09$  s. There was significant positive correlation between the time spent on the rotarod and volume ratio. The animals with large lesions and longer exposition time spent less time on the rotarod compared to animals with small lesions and exposed to laser light for shorter time.

#### *Bar holding*

Strength and grip of both fore- and hind-limbs was assessed in this test. A significant main effect of photothrombotic lesion on the time spent on the bar was demonstrated ( $F(3, 74) = 10.79, p < 0.001$ ). The time spent holding the bar was significantly shorter in both groups of rats subjected to stroke; rats with shorter exposition time ( $16.3 \pm 2.2$  s) and rats with longer exposition time ( $6.2 \pm 0.65$  s) compared to both sham-operated ( $19.5 \pm 2.5$  s) and intact controls ( $22.95 \pm 1.89$  s).

#### *Inclined grid*

Typically, geostatic abilities, immobility, catalepsy and the latencies to turn to  $180^\circ$  as function of time spend on a grid that is incrementally raised to increasing angles is assessed. At present the latency of rats to turn  $180^\circ$  on an inclined grid was recorded for a maximum of 60 s. These

latencies significantly increased in both groups subjected to stroke ( $F(3, 74) = 10.78, p < 0.001$ ), with values of ( $29.16 \pm 3.79$ s) in rats with longer exposition times and ( $20.23 \pm 2.5$ s) in those with shorter exposition times as compared to both intact ( $9.4 \pm 1.4$ s) and ( $6.9 \pm 0.8$ s) sham-operated controls. The animals with longer exposition time had an increased latency to turning on the grid compared to the animals exposed for shorter time to laser light.

### *Ladder rung walking*

The time to complete ladder rung walking task was assessed in a trial with regular gap and another trial with irregular gap arrangement. In this test, 95% of intact controls, 83% of sham-operated rats, 68% of BR\_30 s and 50% of BR\_5 min animals crossed the runway with regular gap pattern within 60 s ( $\text{Chi}^2 = 9.4, p = 0.02$ ). In the trial using irregular gap pattern, 70% controls, 75% sham-operated animals, 37% BR\_30 s and 41% BR\_5 min group completed the task within 60 s ( $\text{Chi}^2 = 10.3, p = 0.06$ ). The ANOVA showed that the latency to cross the ladder with regular gaps was affected ( $F(3, 57) = 3.67, p = 0.017$ ). Surprisingly, there were no statistically significant differences in the latency to cross the ladder between tested groups in both trials with regular or irregular gaps ( $F(3, 57) = 3.67, p = 0.017$ ;  $F(3, 57) = 1.74, p = 0.017$ ) respectively.

### *Open field*

Locomotor and exploratory behavior in rats placed in an open field arena was monitored for five minutes per animal. The distance moved in the OF revealed significant main effect of lesion size on locomotion in the animals subjected to stroke ( $F(3, 57) = 13.69, p < 0.001$ ). The post hoc test showed that only the animals with longer exposition time walked a shorter distance ( $791.4 \pm 77.5$ cm) compared to all other groups; no significant differences were found between the animals

with the small lesions ( $2097.8 \pm 182.1$ cm) and both control groups (sham-operated -  $2015. \pm 185.3$ cm and intact controls -  $2378.0 \pm 144.4$ cm).

## **Discussion**

The effect of cerebral ischemia has been analyzed by many authors, mostly assessing acute and chronic outcomes of lesions induced in adult animals, but rarely the impact of stroke induced in immature animals, and assessed in adulthood. Several reports showed that the immature brain reacts differently to ischemia than does the mature brain, for a review see (Aden et al. 2002; Saucier et al. 2007; Matsumori et al. 2006). Therefore, it is critical to use experimental models of an appropriate age (Aden et al. 2002). Linder et al. and Bona et al. (1997), stressed the importance of long-term functional outcomes, to improve the validity of animal models of stroke rather than relying exclusively on acute consequences.

The present study investigated sensorimotor performances and locomotor activity of adult animals subjected to focal photothrombotic stroke at an early (P7) age. We adopted a technique developed initially for use in neonatal mice (Maxwell and Dyck 2005) to induce photothrombotic stroke in immature rats. Firstly, we focused on assessing whether early postnatal exposure to short and long laser light exposition time induces photothrombotic stroke of two different infarct sizes in adulthood, and secondly, how they are related to sensorimotor performances in adulthood.

To date, the most frequently used neonatal stroke model of long-term neurobehavioral and functional outcomes is hypoxia-ischemia (HI) and middle cerebral artery occlusion (MCA-O) (Almli et al. 2000; Bona et al. 1997; Arteni et al. 2003; Balduini et al. 2000; Balduini et al. 2003; Northington 2006; Renolleau et al. 1998). These models were reported to have high mortality

rates (Maxwell and Dyck 2005) and affect both animal's body weight and behavior (F. Josef van der Staay 1998). For instance in a study by (Renolleau et al. 1998) all 7-day-old pups subjected to left MCA electrocoagulation with transient carotid occlusion lasting for more than 1 hour died during procedure; if occlusion lasted an hour or less, only 10 % of pups died during the first 2 hours. In the present study, all animals survived until adulthood except two animals that died during surgery. Moreover, no differences in animal body weight that might affect motor performances were observed between the controls and animals subjected to stroke (data not shown).

Data from this study depicted two kinds of lesions; different in shape and size relative to laser light illumination. Exposure to this light for 30 sec resulted in small lesion restricted to the sensorimotor cortex leaving the subcortical structures intact. While exposure for 5 min resulted in large lesions that involved all cortical layers including the underlying white matter and striatum of affected hemisphere. The large ischemic lesions had significantly less tissue remaining relative to the group with the small lesions. Sensorimotor ability (motor coordination), includes all processes that affects the brain's ability to synchronize the function of interrelated muscles. Synchronization of muscle movement occurs primarily in the cerebellum. The cerebral cortex is important in controlling the forelimbs and less important in control of the predominantly ambulatory hind-limb. Rats with destroyed cerebellum or inferior olivary complex are unable to perform complex sensorimotor tasks (Rondi-Reig 1997). In this study, motor coordination was measured as a function of the total time each rat was able to remain on a rotating cylinder or the time rats were able to turn to 180° on an inclined platform as the angle of inclination was progressively increased. Our results revealed that sensorimotor performance of rats post stroke was clearly affected when compared to controls. In the bar holding test, one of

the most demanding tests for motor coordination and strength, impairments of motor performance were more expressed. Specifically, the animals with larger lesions were only able to grasp the bar for a very short time.

In the ladder rank walking test, arranged with a pattern of regular gaps, revealed no statistical differences in percentage to complete the task within 60 s among the tested groups. Similar results were also found in the task with irregular pattern. However, the percentage of animals among these groups that successfully completed the given task was decreased to a large extent. These results might be due to motor deficits coupled with decreased motivation to rejoin their cage mates.

In the open field test, animals with larger lesion walked a shorter distance compared to the other groups, suggesting a marked inhibition of locomotion. Surprisingly, among our animals, no signs of ataxia or loss of balance was observed. However, there was a clear relationship between sensorimotor impairments and lesion extent. These outcomes are contrary to previous observations that show no relationship between OF behavior and lesion severity (Chou et al. 2001). On the other hand, our results are in agreement with previous findings indicating that the animals exposed to laser for a longer time had the largest lesions and demonstrated the greatest sensorimotor and locomotion impairments (Sulejczak et al. 2007; Wood et al. 1996). In a combined analysis of controls and HI-subjected rats, a strong correlation between the degree of brain damage and long-term sensorimotor deficits was demonstrated (Bona et al. 1997). In their study, neurological functions were evaluated in a set of four sensorimotor tests performed between five and six weeks after hypoxia-ischemia; asymmetries of limb placing, foot-faults and abnormality in the postural reflex tests were detected. Other studies noted particular differences between behavioral outcomes and lesion size; Jones and colleagues (2008), observed for

example, large unilateral lesions in HI rats accompanied by reduction in forelimb grasping strength, increased number of foot faults in grid-walking test and forelimb asymmetry in cylinder test compared with sham-operated controls. On the other hand, Shanina et al. (2006) demonstrated that small lesions with intact subcortical layers resulted in significant sensorimotor impairments detectable only during the first two weeks after photothrombotic ischemic stroke. Similarly, Biernaskie and colleagues (2005) demonstrated that, the degree of behavioral recovery in animals treated with lidocaine after endotelin-1 induced stroke was dependent on the infarct size. In their study, animals with large lesions were unable to retrieve food pellets; whereas animals with small lesions returned to pre-ischemia performance levels. Our results generated from a photothrombotic model are in accordance with the data described above.

In conclusion, we demonstrated that photothrombotic cerebral ischemic stroke induced in the early postnatal period and tested in adult rats, indeed influenced sensorimotor performance governed by the affected brain region. This model makes it possible to mimic lesion variability, size and location as observed in most human stroke; providing an opportunity for long-term evaluation of behavioral and neuropathological endpoints. Future considerations may therefore be aimed at exploring and refining basic experimental issues; it will be of great interest to extend this study to analyze kinematic changes and task performance such as limb placing, foot faults and postural motor function in this particular model of stroke.

### **Conflict of Interest**

There is no conflict of interest.



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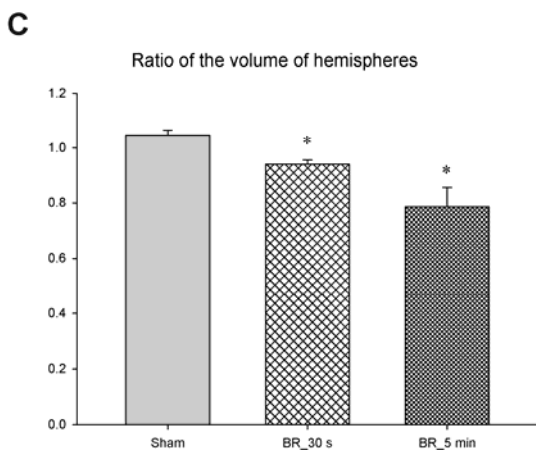
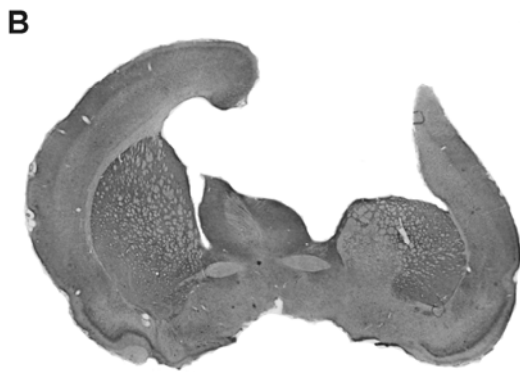
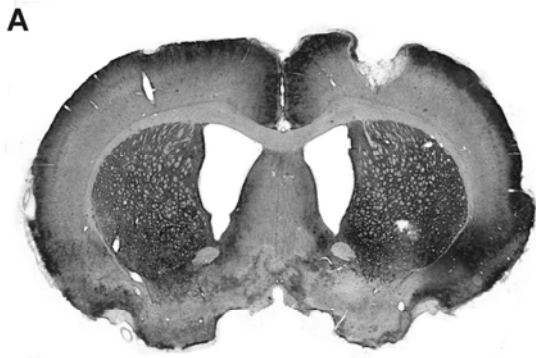
### **Text to Figures**

**Fig.1** Representative, Nissl-stained coronal brain section of photothrombotic infarct induced in P7 and assessed in adult rats showing, the involvement of all cortical layers in BR\_30 s (A) and BR\_5 min (B) including the underlying subcortical white matter. Sections obtained at 50µm intervals throughout the lesion were used to calculate volume ratio (C) of hemispheres of sham-operated controls, BR\_30 s and BR\_5 min.

**Fig.2.** Effects of photothrombosis induced at P7 on the sensorimotor behavior of adult rats; Abscissa: C (controls); Sham (sham operated controls); BR\_30 s (group exposed to laser light for

30 s); and BR\_5 min (group exposed to laser light for 5 min). Ordinate: mean + S.E.M.,

\*Compared to both C and Sham, #Compared to Sham, +Compared to BR-30 s.



**Figure 1**

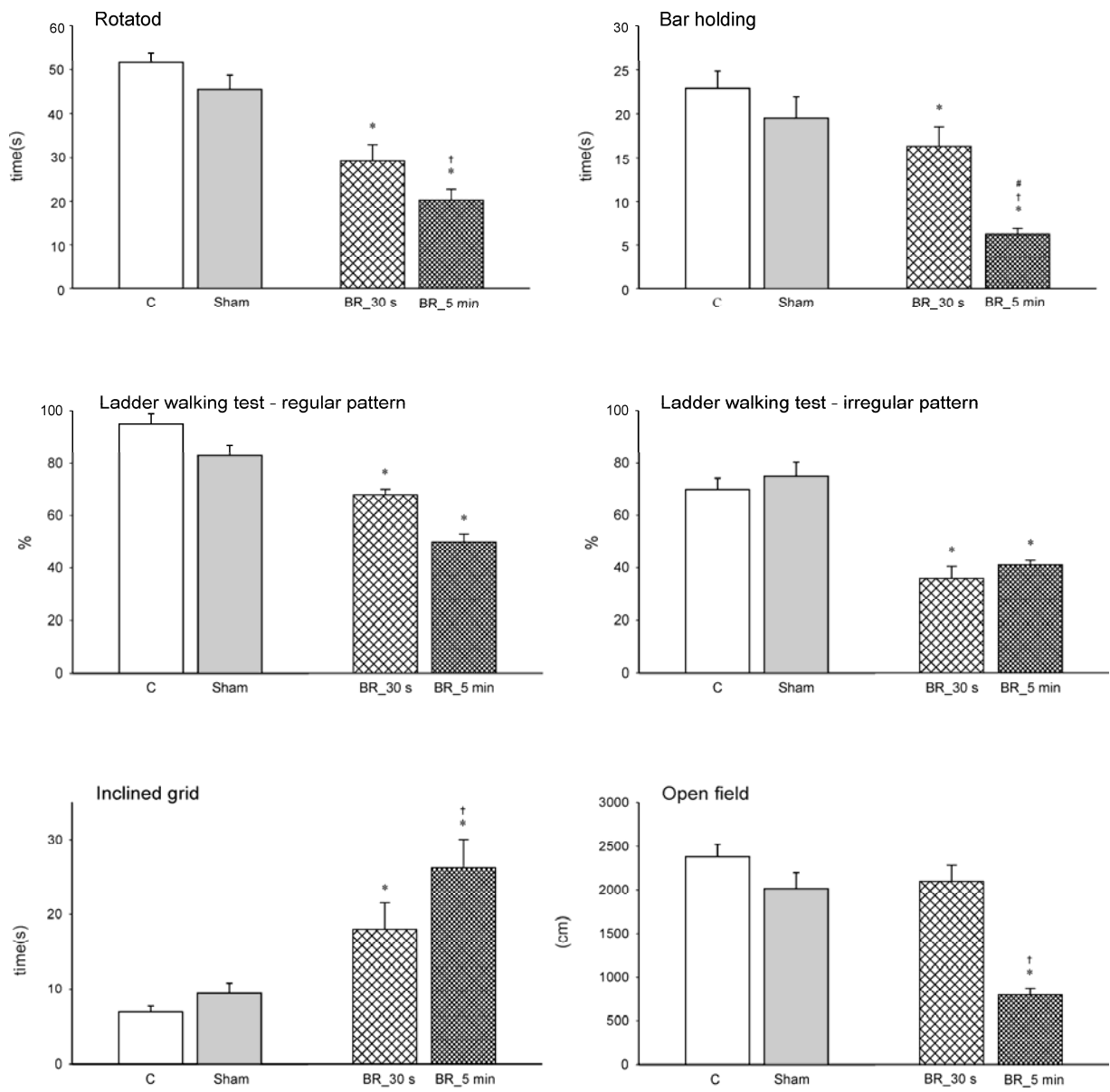


Figure 2