Histological analyses

Morphological evaluation of the brain injury was performed, as described previously (Tsuji et al., 2004, 2012). Forty-eight hours after the MCAO or HI insult, the brain was removed and sectioned coronally in 1-mm thick slices. The area of the viable ipsilateral and contralateral hemispheres, which stained red with 2,3,5-triphenyltetrazolium chloride (TTC) in each brain section, was measured using ImageJ software (NIH, Bethesda, USA). The hemispheric volume was estimated by integrating the hemispheric areas.

For longer-term evaluation, separate sets of animals were perfusion-fixed intracardially with 4% paraformaldehyde, 8 weeks after the insult. In assessing the hematoxylin–eosin-stained sections, neuropathological injury in the cerebral cortex was scored on a scale ranging from 0 to 4 points (0, no injury; 4, extensive confluent infarction). Neuropathologic injury in the hippocampus, striatum, and thalamus was scored on a scale ranging from 0 to 6 points. The ipsilateral and contralateral areas in the four regions and the corpus callosum were measured using ImageJ software. The ratios of the ipsilateral/contralateral areas in the five regions were calculated after summing the areas in four brain sections (cortex) or two brain sections (hippocampus, striatum, thalamus, and corpus callosum).

Statistics

The mortality rate of the animals was analyzed using Fisher's exact test with Bonferroni's correction for multiple comparisons. Hemispheric volumes, and CBF were assessed using two-way analysis of variance (ANOVA), followed by the Bonferroni test. The differences in body weight were assessed using one-way ANOVA, followed by the Bonferroni test. The injury scores were not distributed normally, so differences in injury scores were assessed with the Mann–Whitney *U* test. Ratios of the ipsilateral/contralateral areas were assessed using a Kruskal–Wallis test, followed by Dunn's multiple comparison, as the variances of the ratios were significantly different among the three groups. Pearson's product–moment correlation coefficient analysis was performed to determine the correlation between CBF and brain injury. Outcomes in the rotarod and open-field tests performed at two time points were assessed using two-way repeated measures ANOVA. Temporal changes during the course of a 60-min session in open-field test were then analyzed using two-way repeated measures ANOVA. Differences were considered significant at $P < 0.05$. The results are presented as the mean ± standard deviation (SD), unless otherwise noted.

Results

Mortality and body weight

All pups that were prepared for surgery underwent the surgery successfully. Although some pups experienced bleeding during the MCAO surgery, all pups were included in the subsequent analyses. Survival was 100% at 48 h and 85% at 8 weeks after MCAO (Table 1). Body weights at P12 and 8 weeks later did not differ among groups, including the no-surgery controls (Table 2).

Table 1
Mortality rates.

	48 h-survival cohort	8-week-survival cohort
No-surgery		0/13
Sham-surgery		2/17
HI	1/12	6/22
MCAO	0/10	3/20

None of the pups died during the surgical procedure for either MCAO (middle cerebral artery occlusion) or HI (hypoxia–ischemia). In each cohort, mortality rates did not differ significantly between groups.

Table 2
Body weights.

	Postnatal day 12	8 weeks later
No-surgery	6.5 ± 0.6	21.9 ± 2.0
Sham-surgery	6.9 ± 0.9	22.2 ± 2.1
HI	6.6 ± 1.4	20.5 ± 2.3
MCAO	6.8 ± 1.1	21.9 ± 3.2

Body weights (grams) (mean ± SD) at postnatal day 12 (the day of surgery) and 8 weeks later were not different between groups. MCAO; middle cerebral artery occlusion, HI; hypoxia–ischemia.

Morphological brain injury

Forty-eight hours after the insult, moderate-complete TTC discoloration was observed in all 10 pups that were subjected to MCAO, while discoloration was observed in only five out of 11 pups that were subjected to HI (Fig. 2A). The discoloration was confined to the ipsilateral cerebral cortex, and its location and size were consistent in all pups in the MCAO group, with the exception of one pup that exhibited discoloration extending to the striatum. In contrast, the location and size of the discoloration was markedly more variable in the HI group. The mean % stroke volume was 25.1 ± 3.6% in the MCAO group and 15.5 ± 18.6% in the HI group. The % stroke volume was calculated as follow: ((contralateral volume − viable ipsilateral volume) / contralateral volume) × 100%. Variances of the viable ipsilateral hemispheric volume and % stroke volume differed significantly between the two models ($P < 0.001$) (Fig. 2B).

Eight weeks after the insult, all 17 mice with MCAO exhibited consistent macroscopic cortical damage (Fig. 2C). The mean ipsilateral hemispheric volume was 73.0 ± 3.2 mm^3 in the MCAO group, and 72.3 ± 23.0 mm^3 in the HI group (Fig. 2D). Of note, the sham-surgery group was not different from the no-surgery group, suggesting that the open-skull surgical procedure did not cause noticeable morphological damage. No sex differences in hemispheric volumes were observed at either time point in any of the groups.

Neuropathological injury scores in the four brain regions examined differed between the two models (Fig. 3A). The ratios of the ipsilateral/contralateral areas in the four regions and corpus callosum differed among the three groups including the sham-surgery group (Fig. 3B). Interestingly, in the MCAO group, most mice exhibited mild thalamic injury, in contrast with a virtual absence of striatal or hippocampal injury. Furthermore, the thalamic damage in the MCAO model was strictly restricted to the ipsilateral ventroposterior thalamic nuclei (VPN), which contained many pyknotic cells (Fig. 3C). In contrast, the thalamic injury in the HI model was variable in terms of its distribution and severity. In both models, the ispilateral corpus callosum exhibited mild atrophy; however, this only reached statistical significance in the MCAO model.

CBF

The CBF was decreased in the MCA territory on the ipsilateral side in all pups 24 h after the HI or MCAO insult. The degree of the CBF reduction was consistent after MCAO, whereas it was variable between animals after HI (Figs. 4A, B). The CBF 24 h after the insult was compared with the morphological brain injury at 8 weeks after the insult (Fig. 4C). The reduction in CBF after the MCAO did not correlate with the subsequent morphological brain injury. In stark contrast, the reduction in CBF after the HI insult correlated strongly with brain injury ($R^2 = 0.99$), which is consistent with our previous report in P8 mice with the HI insult (Ohshima et al., 2012).

Rotarod performance

Sensorimotor performance, as assessed by rotarod treadmill at 2 and 7 weeks after the insult was analyzed by two-way repeated

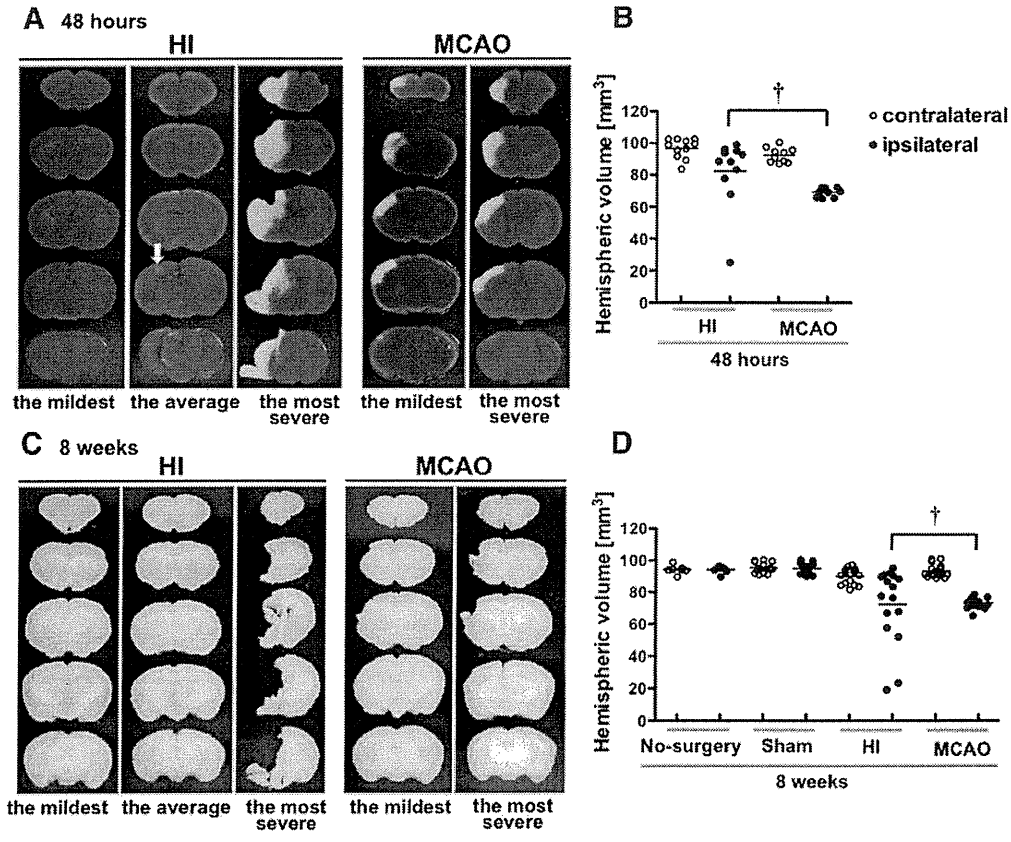

Fig. 2. Macroscopic brain injuries. (A) Images of TTC-stained brain sections 48 h after middle cerebral artery occlusion (MCAO) or hypoxia–ischemia (HI). The brains with the mildest injury and the most severe injury in the MCAO group and those with the mildest, the average, and the most severe injury in HI group are shown. The brain injury was highly consistent after MCAO. In contrast, the brain injury varied substantially after HI (the arrow indicates a small area of discoloration). (B) Hemispheric volumes of viable tissue, which stained red, examined at 48 h after the insult (HI $n = 11$; MCAO $n = 10$). (C) Images of brain slices 8 weeks after the insult. (D) Hemispheric volumes examined at 8 weeks after the insult. † Significant difference in the variances between the groups ($P < 0.001$). There were no significant differences in the ipsilateral hemispheric volumes between the no-surgery and sham-surgery groups, nor in the contralateral hemispheric volumes in the no-surgery, sham-surgery group, and MCAO groups. (no-surgery $n = 7$; sham-surgery $n = 15$; HI $n = 16$; MCAO $n = 17$).

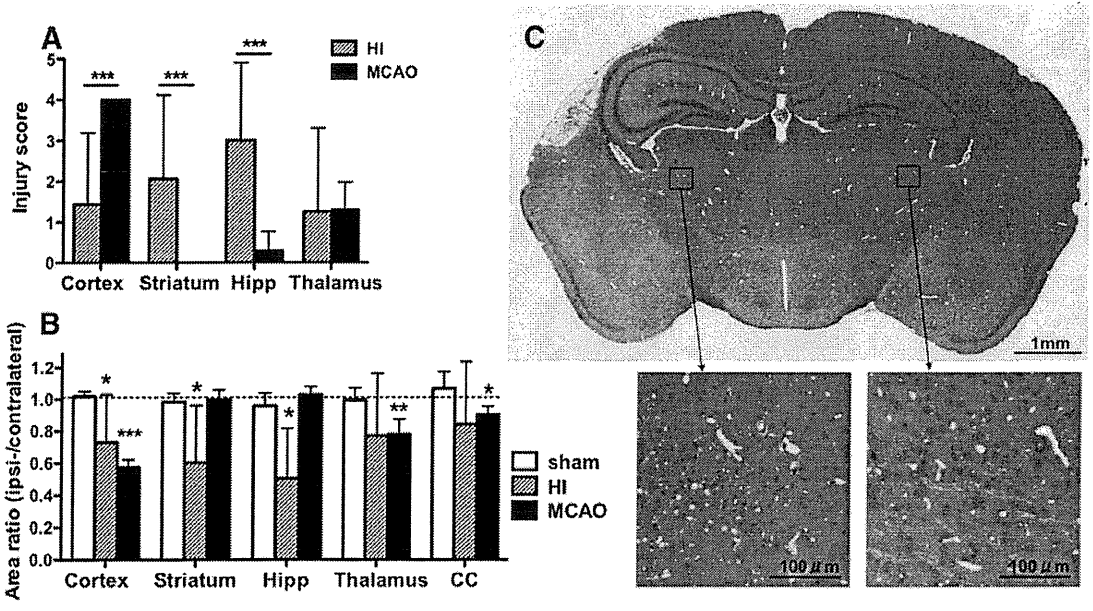

Fig. 3. Microscopic brain injuries. (A) Neuropathological injury scores examined in hematoxylin–eosin-stained sections 8 weeks after the insult. ***$P < 0.001$. (HI $n = 16$; MCAO $n = 17$) (B) The ratios of ipsilateral/contralateral areas in each region examined at 8 weeks after the insult. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, compared with sham. Note the difference in the error bars (standard deviation) between the models (sham-surgery $n = 7$; HI $n = 10$; MCAO $n = 10$). Hipp; hippocampus. CC; corpus callosum. (C) Representative image of H&E-stained sections of mice brain 8 weeks after the MCAO. There is a clearly demarcated old infarct in the ipsilateral cortex. The ipsilateral thalamus is mildly atrophic. The labeled boxes indicate the regions that were selected for higher magnification (×20). Many pyknotic neurons are observed in the ipsilateral ventroposterior thalamic nucleus (VPN). The contralateral VPN appears normal.

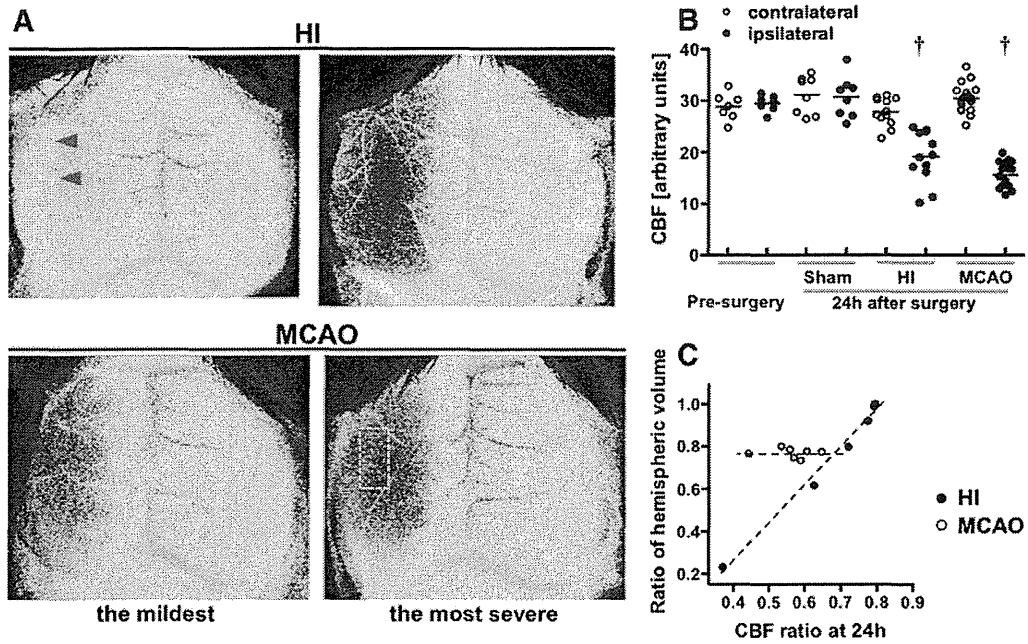

Fig. 4. Cerebral blood flow. (A) Images of the cerebral blood flow (CBF) 24 h after the insult. The reduction of the CBF, indicated by the bluish color, was consistent after MCAO, but not after HI (the arrowheads indicate the main trunk of the MCA). (B) CBF levels were measured in the ischemic core region (the box with dotted line) of the MCA territory and in the matching region on the contralateral side before and after the insult. † Significant difference compared with the pre-surgery or sham-surgery groups ($P < 0.001$), and significant difference between each model ($P < 0.01$) (pre-surgery $n = 7$; sham-surgery $n = 8$; HI $n = 12$; MCAO $n = 17$). (C) The ratio of the ipsilateral CBF to the contralateral CBF at 24 h after the insult was compared with the ratio of the ipsilateral hemispheric volume to the contralateral hemispheric volume (assessed 8 weeks after the insult). The correlation between the degree of CBF reduction and the degree of brain damage is extremely strong in the HI group ($R^2 = 0.99$). (HI $n = 6$; MCAO $n = 7$).

measure ANOVA. There were significant time and group differences; the performance in mice with MCAO was significantly impaired compared with that in the sham-surgery group (Fig. 5). The impairment in the rotarod performance in mice with HI was not statistically significant.

Open-field activities

We initially analyzed overall activities during 60-min sessions at 5 and 7 weeks after the insult using two-way repeated measures ANOVA (Figs. 6A, B). While there was no time difference with respect to either locomotion or rearing, there was a significant group difference with respect to rearing, but not locomotion; mice with HI were hypoactive compared with the mice in the other three groups. There were no overall reductions in locomotion or rearing in the mice with MCAO.

We then analyzed the temporal changes throughout a 60-min session in 5-min increments using two-way repeated measures ANOVA. With respect to locomotion, the mice with MCAO did not respond to the change of environment from light to dark, whereas mice in all other groups became hyperactive in response to the dark environment, either at 5 weeks (data not shown) or 7 weeks after the insult (Fig. 6B). With respect to rearing, there were significant group differences at both 5 weeks (data not shown) and 7 weeks (Fig. 6C) after the insult. The mice with HI exhibited significantly less rearing compared with mice in all other groups.

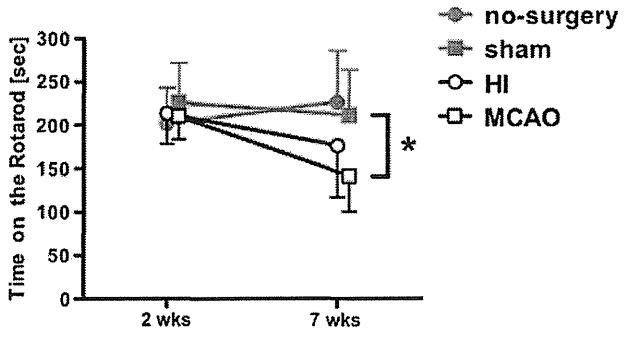

Fig. 5. Rotarod test. Repeated-measures two-way ANOVA showed significant time and group differences in sensorimotor performance, assessed 2 and 7 weeks after the insult. Performance was significantly impaired in mice with MCAO compared with the sham-surgery groups. *$P < 0.05$. (no-surgery $n = 19$; sham-surgery $n = 13$; HI $n = 16$; MCAO $n = 11$, 2 weeks after the insult. no-surgery $n = 9$; sham-surgery $n = 10$; HI $n = 13$; MCAO $n = 11$, 7 weeks after the insult).

Discussion

In this study, we have demonstrated that permanent occlusion of the MCA in CB-17 mice induces a highly reproducible and selective cortical infarction. We believe that our model has clinical relevance to, at least a portion of infants with stroke, as an isolated large infarct in the vascular territory of left MCA is most commonly observed in infants with stroke (Lee et al., 2005; Sreenan et al., 2000). This high degree of consistency allows the effective screening of various experimental treatments using smaller numbers of animals. The most important point in achieving this high reproducibility is the use of the CB-17 strain, which exhibits very little variation in the cerebral vascular structure (Taguchi et al., 2010). It is known that the degree of brain damage and its reproducibility in neonatal rodent models of HI and stroke are dependent upon the strain used (Comi et al., 2005; Sheldon et al., 1998). In addition to the high reproducibility, the advantages of our model are its simple procedure and high long-term survival, which provides the opportunity for long-term evaluation of neuropathological and functional outcomes. Indeed, our model exhibited significant long-term neurofunctional deficits.

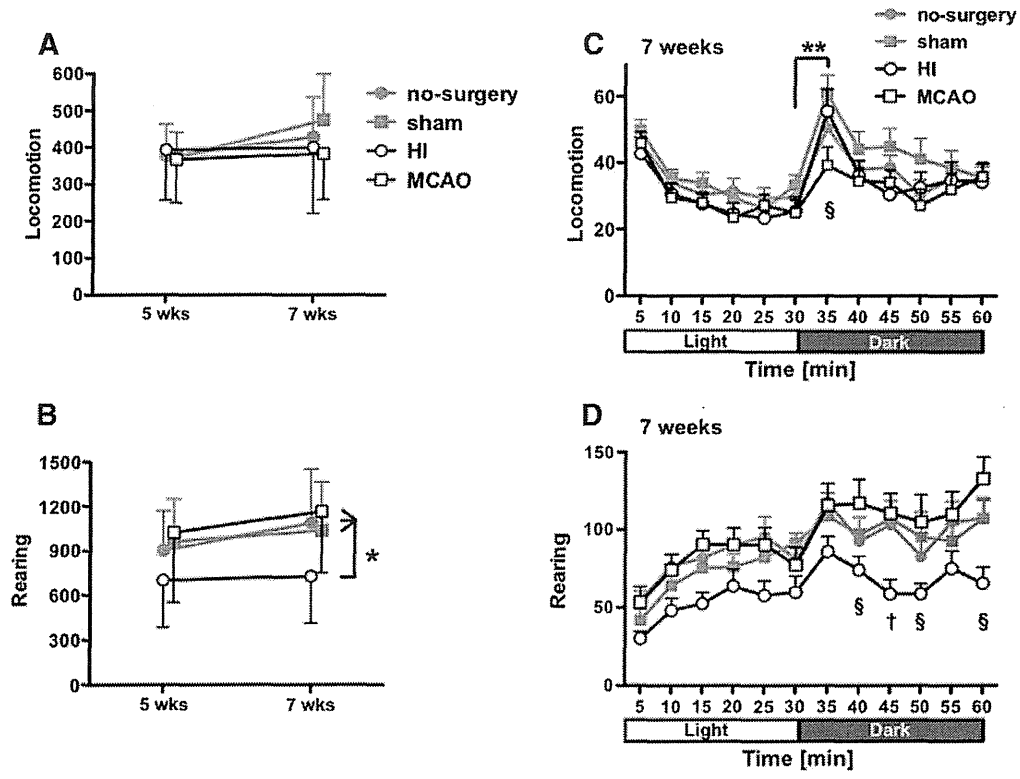

Fig. 6. Open-field test. (A, B) Overall activities during the 60-min session 5 and 7 weeks after the insult were analyzed by two-way repeated measures ANOVA. While there was no time difference with respect to either locomotion or rearing, there were significant group differences with respect to rearing, but not locomotion; mice with HI were significantly hypoactive compared with mice in the other three groups. (no-surgery $n = 14$; sham-surgery $n = 10$; HI $n = 16$; MCAO $n = 14$, 5 weeks after the insult. no-surgery $n = 13$; sham-surgery $n = 11$; HI $n = 16$; MCAO $n = 13$, 7 weeks after the insult). (C, D) Temporal changes in 5-min increments were analyzed by repeated-measures two-way ANOVA. There were significant group differences with respect to locomotion at 7 weeks after the insult. Mice in the MCAO group were significantly hypoactive during the first 5-min period in the dark than mice in the HI group. § $P < 0.05$. There were significant increases in the activity from the last 5-min period in the light environment to the first period in the dark environment in all groups except for the MCAO group. ** $P < 0.01$. With respect to rearing, there were significant group differences at 7 weeks. Mice in the HI group exhibited significantly less rearing activity. § $P < 0.05$, compared with MCAO group, † $P < 0.05$, compared with the no-surgery, sham-surgery and MCAO groups. Mean ± SEM.

Six models of neonatal stroke using artery obstruction have been developed (Ashwal et al., 1995, 2007; Bonnin et al., 2011; Comi et al., 2004; Derugin et al., 1998, 2000; Mitsufuji et al., 1996; Renolleau et al., 1998; Wen et al., 2004), and are summarized in Table 3. All models, except one, exhibit obvious inter-animal variability; some of the animals subjected to the insult do not develop infarct, as is the case in the HI model. In a permanent MCAO model developed by Wen et al. (2004), in which a tailor-made intraluminal suture embolus was placed in P7 SD rats, infarct was noted in all 10 pups that were subjected to the insult. However, the long-term survival was not reported. Taken together, among the currently available rodent models of neonatal stroke our model exhibits the highest reproducibility with excellent long-term survival. Nevertheless, those models, including ours, should be complementary, in order to lead to new understanding of the mechanisms of neonatal stroke and to find therapies for neonatal stroke. Our model has some weaknesses compared with other models. Firstly, this model does not utilize a reperfusion phase. Reperfusion may or may not occur in some patients, or the reperfusion may occur too late to activate its downstream events in other patients. Secondly, increasing or decreasing the degree of brain injury is not possible in this model. Thirdly, craniotomy results in stress to the animal and trauma to local tissues, even though the present study

Table 3
Immature rodent models of cerebral ischemia.

	Method of obstruction	Age and Species/strain	Ratio of infarct formation*	Long-term survival	Author and reference
1	t-f-MCAO	P14-18 or P10 SH rats	8/9	21% by 28 days	Ashwal et al., 1995, 2007
2	t-f-MCAO	P7 Sprague–Dawley rats	8/10, 20/31	71% by 7 days	Derugin et al., 1998, 2000
3	p-CCAO + t-CCAO†	P10 Wistar rats	NA	NA	Mitsufuji et al., 1996
4	p-MCAO + t-CCAO‡	P7 Wistar rats	10/10, 36/66	NA	Renolleau et al., 1998; Bonnin et al., 2011
5	p-CCAO	P12 CD1 mice	20/28	86% by 7 days	Comi et al., 2004
6	p-f-MCAO	P7 SD rats	10/10	NA	Wen et al., 2004
Present study	p-MCAO	P12 CB-17 mice	27/27	85% by 8 weeks	

These are unilateral cerebral ischemia models, unless otherwise noted. t-; transient. f-; intraluminal filament. p-; permanent. MCAO; middle cerebral artery occlusion. CCAO; common carotid artery occlusion. P; postnatal day. SH; spontaneously hypertensive. NA; not available. * Ratio of the number of animals presenting with obvious infarct to the number of animals that survived until the time of assessment. † Unilateral p-CCAO combined with contralateral t-CCAO. ‡ Unilateral MCAO by electrocoagulation combined with ipsilateral t-CCAO.

demonstrated that sham-surgery operated mice were not different from the no-surgery control mice, with respect to brain morphology, CBF, and behavior.

The differences in the variability between the two models (i.e., MCAO and HI) demonstrated in our study can provide insights into the mechanisms that lead to extensively variable susceptibility to HI insult by animals, even within littermates. The pivotal cause of the variation remains poorly understood. A number of explanations have been proposed for inter-animal variations in the extent of brain damage; 1) differences in collateral arteries in the brain (Rubino and Young, 1988), 2) the existence of several major MCA branching patterns (Rubino and Young, 1988), 3) subtle differences in the genetic background, 4) blood sugar level differences, which may result from variations in feeding times and amount (Chen et al., 2011; Hattori and Wasterlain, 1990), 5) temperature variation, 6) weight variation (Menzies et al., 1992), and 7) long surgery time and duration of isoflurane exposure (Chen et al., 2011). Our contrasting results in the two models suggest that these explanations are unlikely, because only the HI model exhibited substantial variability, despite the fact that all the aforementioned factors were consistent for both the MCAO and HI models. We cannot exclude the possibility that structural and physiological variations in the circle of Willis could contribute to the inconsistent brain damage after HI. Bonnin et al. (2011) reported that establishment of collateral recruitment via the basilar artery led to the presence or absence of a lesion. We also cannot exclude other possibilities, such as differences in the susceptibility to reperfusion damage, or in cardiovascular and respiratory function. As our model and the above-mentioned reproducible stroke model (Wen et al., 2004) are both permanent occlusion models, some mechanisms that occur during reperfusion may lead to large inter-animal variability.

There has only been one previous study in the literature that directly compared the MCAO and HI models (Ashwal et al., 2007). Unlike ours, variability in brain injury did not appear to be different between the two models in the previous study. The discrepancy between their results and ours may be due to the different MCAO procedures and the animals used. The previous report used a transient MCAO model in P10 spontaneously hypertensive rats, whereas we used a permanent MCAO model in P12 CB-17 mice.

We observed thalamic damage that was confined to the ipsilateral VPN in our MCAO model. As the VPN is supplied by thalamoperforating arteries originating from the basilar artery systems (Oscar and Holschneider, 2012), MCAO does not cause direct ischemic injury to this nucleus. Secondary neuronal damage in the thalamic nuclei after focal ischemia has been reported in adult rat models (Dihne et al., 2002; Schroeter et al., 2006). The damage in VPN was possibly due to retrograde degeneration of the thalamocortical projection (Dihne et al., 2002). Thalamic atrophy has been seen in children with neonatal MCA infarct (Giroud et al., 1995).

Our MCAO model exhibited neurological dysfunction in the rotarod and open-field tests; the mice with MCAO lost the response to a change of the environment from light to dark, while their overall activities were not disturbed significantly. The results in behavioral tests in immature rodent models of stroke or HI are not consistent and can often be contradictory. Rodents with ischemic insult exhibited significantly poorer rotarod performance compared with controls in some (Chen et al., 2012; Jansen and Low, 1996), but not all studies (Aden et al., 2003; Kadam et al., 2009; Lubics et al., 2005). Similarly, rodents with ischemic insult exhibited altered behavior in open-field test in some studies (Aden et al., 2002; Kadam et al., 2009; Lubics et al., 2005), but not in others (de Paula et al., 2009). The discrepancies among the reports may be due to differences in species/strain (de Visser et al., 2006), in the extent of brain damage, in the timing of the assessment (Lubics et al., 2005), and in the experimental paradigm. In the future more sensitive measures will be needed to confirm these results.

Seizure behavior, which is one of the main presenting symptoms in neonates with stroke, was not observed in our model during the 2-hour period following artery occlusion. Seizure behavior has been reported in a stroke model in immature CD1 mice (Comi et al., 2004), but not in other stroke models in immature rodents. That is likely due to strain-related differences in the susceptibility to seizures (Comi et al., 2005) or simply due to a lack of detailed assessment for seizure activities in the models. One possible reason to explain the inability to cause seizure in our model would be the distribution of the brain injury, which is confined to the ipsilateral cortex and did not involve the hippocampus. More detailed and longer observation periods will be needed before we can conclude that our model does not cause seizure activity, as the median time to seizure after the insult can be more than 2 h in some strains (Comi et al., 2005).

Conclusions

We believe that this model is useful for detailed analyses in preclinical studies of neonatal stroke using a smaller number of animals, because of its high reproducibility, excellent long-term survival rate, and measurable neurofunctional deficits, and that this model will be useful in assessing functional improvement in response to experimental therapies.

Disclosures

None.

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Letter to the Editor

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Letter by Taguchi et al Regarding Article, "Granulocyte Colony-Stimulating Factor in Patients With Acute Ischemic Stroke: Results of the AX200 for Ischemic Stroke Trial"

To the Editor:

We read with interest a recent article by Ringelstein et al.[1] The authors described the results of a phase 2B clinical trial of granulocyte colony-stimulating factor (G-CSF) for patients with stroke, which did not result in any beneficial effects. The authors concluded that the failure of this clinical trial was mainly attributable to the problem of translating findings from the animal laboratory to patients with clinical stroke and thus raised the question, "Are rodent models questionable for predicting stroke drug efficacy in humans?" However, we think that the negative results of this clinical trial had been predictable from previous results of basic experiments with animal models.

The clinical trial was designed on the basis of a meta-analysis of experimental models of cerebral ischemia.[2] However, in the majority of cases, the experiments that were included were of transient cerebral ischemia ranging from 1 to 3 hours. Transient ischemia is a model of ischemia-reperfusion injury, and the therapeutic target is mainly protection from apoptotic neural cell death caused by oxidative stress after reperfusion. In contrast, most cases of human stroke show massive necrotic neural cell death caused by a poor supply of cerebral blood flow. Although, in the end, neural cell death is observed in both types of ischemia, the actual pathological states and therapeutic targets are different from each other, and the discrepancy between apoptotic and necrotic cell death should be considered when the clinical trial is designed. We point out that we clearly demonstrated that G-CSF has a negative effect on stroke outcome using a permanent cerebral artery ligation model in immunocompetent mice, and we drew attention to the discrepancy between transient and permanent cerebral occlusion models.[3] Furthermore, activated granulocyte, which is significantly mobilized by G-CSF from bone marrow to peripheral blood, is well known to enhance brain damage in experimental stroke model through enhancing inflammation at the site of cerebral ischemia.[4] Therefore, it is not surprising that the results of the clinical study show that G-CSF has no therapeutic effect on the outcome of patients with stroke, and the results are not dissimilar to previous findings shown in an experimental stroke model.

In conclusion, we think that it is not rational to conclude that rodent models are questionable in predicting stroke drug efficacy in humans. With regard to the clinical trial in question, previous findings obtained by basic experiments had predicted the negative effect of G-CSF on stroke outcomes (ie, other than experiments with transient ischemia models that mimic patients who had reperfusion injury after recanalization; however, this is not the case with majority of patients with stroke). We think that rodent models are useful for human trials as long as the therapeutic target and the patients enrolled are given proper consideration when the clinical trial is first designed.

Disclosures

None.

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