Effects of cerebral ischemia in mice lacking DNA methyltransferase 1 in post-mitotic neurons

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DNA methylation is important for controlling gene expression and is catalyzed by DNA methyltransferase (Dnmt1) an enzyme abundant in brain. We recently demonstrated that mice expressing reduced levels of Dnmt1 are protected from cerebral ischemia. Here, we used the cre/loxP system to produce conditional mutants that lack Dnmt 1 in postmitotic neurons of the postnatal brain. We demonstrate that animals heterozygous for the conditional allele ($Dnmt1^{1lox/+}$) have significantly smaller infarcts following 1 h middle cerebral artery occlusion/reperfusion compared to their wildtype litters. Surprisingly, mice with a deletion of $Dnmt1$ in post-mitotic neurons ($Dnmt1^{1lox/c}$) were not protected. In conclusion, we demonstrate that reduced levels of Dnmt1, but not its absence, in post-mitotic neurons protect from ischemic brain injury. NeuroReport 12:3763–3766 © 2001 Lippincott Williams & Wilkins.

Key words: Cerebral ischemia; DNA methylation; DNA methyltransferase; Epigenetics; Stroke

INTRODUCTION

Mammalian DNA cytosine methylation is a covalent, post-replicative modification of genomic DNA and influences many cellular events, including gene transcription, differentiation, imprinting and genome stability [1,2]. DNA methylation is catalyzed by three different DNA (cytosine-5) methyltransferases (Dnmts): two *de novo* methyltransferases (Dnmt 3a and 3b) and the maintenance Dnmt 1 (EC 2.1.1.37) [3]. The essential role of Dnmt1 during development was demonstrated using *Dnmt1* mutant mice, which die between embryonic day 8 (E8) and E10.5 [4,5].

Although Dnmt1 is usually down-regulated in non-replicating cells, its activity and expression is surprisingly high in postmitotic neurons implying a neuron-specific role [6,7]. We have recently demonstrated that levels of DNA methylation activity are increased after ischemic brain injury, and that this increase is partly dependent on Dnmt1 activity [8]. Blocking Dnmt1 activities, either genetically or pharmacologically, is protective to the injured neurons, suggesting that a balance of DNA methylation levels is important for neuronal survival [8]. Our previous observations using constitutional *Dnmt1* mutants, however, were limited by the early lethality of homozygous animals [4,5]. Moreover, constitutional deletion cannot distinguish between effects of Dnmt1 during development and effects in the adult brain, nor between neuronal and non-neuronal effects.

To overcome these problems conditional *Dnmt1* mutant mice were generated using the cre/loxP system [9,10]. To enable this, exons 4 and 5 of the dnmt1 gene were flanked by loxP sites [9,10]. To produce animals lacking Dnmt1 in postmitotic CNS neurons CamK-cre transgenic mice were used in which the cre expression is under the control of the neuronal calmodulin-kinase IIα (CamK) promotor. In this study we examined whether there are significant differences in the suceptibility to experimental brain ischemia in mice after conditional deletion of *Dnmt1*.

MATERIALS AND METHODS

Brain-specific* Dnmt1 *conditional mutant mice: We used the cre/loxP binary system to generate *Dnmt1* conditional mutants. Details of generating the *Dnmt1* conditional allele ($Dnmt1^{2lox}$) are reported elsewhere [9,10]. Briefly, in this line of mice exons 4 and 5 of the *Dnmt1* gene were flanked by loxP sites [9,10]. Cre-mediated deletion of exons 4 and 5 would lead to out-of-frame slicing from exon 3 to exon 6, resulting in a null Dnmt1 allele [9]. For conditional gene deletion in postmitotic CNS neurons, we used the CamK-cre transgenic mice in which the cre expression is under control of the neuronal calmodulin-kinase IIα (CamK) promotor. We previously characterized the distribution of cre-mediated gene deletion by crossing the CamK-cre transgenic mice with the lacZ reporter strains [10]. The conditional mutants also carried the previously described

Dnmt1 mutant C-allele which represents a *Dnmt1* null allele [11]. Southern blotting and PCR were used for genotyping as described [10]. In this study we only compared litter mates of the following genotypes: $Dnmt1^{2lox/+}$ (wildtype controls), $Dnmt1^{1lox/+}$, CamK-Cre (heterozygotes) and $Dnmt1^{1lox/c}$, CamK-Cre (conditional mutants).

Mouse model for focal cerebral ischemia: All animal experiments were performed according to NIH and institutional guidelines. Mice (18–22 g) were anesthetized with 1.5% halothane (induction) and maintained on 1.0% halothane in 70% N_2O/30% O_2 by face mask. Focal cerebral ischemia was induced as described [8,12]. In brief, MCA occlusion was produced by inserting a silicone-coated 8-0 monofilament into the internal carotid artery. One hour later the filament was withdrawn to reperfuse the brain. To ensure equivalent levels of ischemia between groups, regional cerebral blood flow (rCBF) was measured by laser Doppler flowmetry using a flexible skull probe [8,12]. In randomly selected animals the left femoral artery was cannulated for arterial blood pressure and blood gas determination [8,12]. Arterial blood samples (50 μl) were analyzed for pH, PaO_2 and $PaCO_2$ using a blood gas/pH analyzer (Corning 178, Ciba-Corning Diagnostics, Medford, MA). Core temperature was maintained at $\sim 37 \pm 0.5°C$ with a thermostat (FHC, Brunswick, ME, USA) and a heating lamp during the monitoring period until 1 h after reperfusion.

Determination of lesion size: Animals were sacrificed at 24 h reperfusion and brains were snap-frozen in isopentane on dry ice for cryostat sectioning. Infarct areas were quantified with an image analysis system (M4, Imaging Research, St. Catharines, Ontario, Canada) on 20 μm hematoxylin/eosin (HE)-stained cryostat sections. Infarction volume was calculated by summing the volumes of each section directly [12] or indirectly using the formula: contralateral hemisphere (mm^3)−undamaged ipsilateral hemisphere (mm^3). The difference between direct and indirect infarct volumes reflects brain swelling. Percent infarct size was calculated as percentage of indirect infarct of total hemisphere volume.

Neurological deficits: Mice were tested for neurological deficits and scored as described by Bederson *et al.* [13] with the following minor modifications: 0 = no observable deficit (normal); 1 = failure to extend the right forepaw (mild); 2 = circling to the contralateral side (moderate); 3 = loss of walking or righting reflex (severe). Animals were rated in a blinded fashion. Assessments were made after 30 min and 24 h.

Data analysis: Experiments (cerebral ischemia, evaluation of infarct size, physiology, neurological deficits) were performed in a blinded fashion. Data are presented as mean ± s.e. Differences between groups were evaluated by ANOVA followed by Tukey's test (infarct size, physiology). Neurological scores were analyzed by Kruskal–Wallis non-parametric ANOVA on ranks. $p < 0.05$ was considered statistically significant.

RESULTS

To determine whether conditional *Dnmt1* mutant mice have altered susceptibility to tissue injury, animals were subjected to 1 h filamentous occlusion of the left middle cerebral artery followed by reperfusion for 23 h. Infarcts were measured on HE-stained cyostat sections (20 μm) and compared between groups. Heterozygous $Dnmt1^{1lox/+}$ mice had significantly smaller infarcts (32%) than wild-type littermate controls ($Dnmt1^{2lox/+}$; 107.1 ± 6.1 vs $72.6 \pm 9.2 \, mm^3$, $p < 0.01$). When infarction volume was corrected for brain swelling (i.e. calculated by the indirect method), infarcts in the heterozygote animals were significantly decreased by 33% (86.4 ± 3.8 vs $55.4 \pm 5.9 \, mm^3$, $p < 0.005$; Fig. 1a). Significantly smaller lesions were evident in four (i.e. sections 2–5) of the five standardized coronal brain sections (Fig. 1b). The differences in infarct size related largely to differences in cortical damage ($64.8 \pm 4.8 \, mm^3$ vs $39.0 \pm 7.6 \, mm^3$, $p < 0.01$), whereas striatal infarction was similar between groups (Fig. 1c).

Surprisingly, however, conditional mutant $Dnmt1^{1lox/c}$ mice were not different from litter mate wildtype controls and infarcts were significantly larger than in heterozygous animals (Fig. 1a–c). Direct infarct size following 1 h MCAo/reperfusion was $103.6 \pm 7.1 \, mm^3$ and indirect infarct size was $81.5 \pm 4.6 \, mm^3$.

All animals exhibited a score of $\geqslant 2$ 30 min after the onset of ischemia and after animals recovered from anesthesia. At 24 h the deficits tended to be lower in the heterozygous $Dnmt1^{1lox/+}$ mice, but the differences between groups did not reach statistical significance (Fig. 1d).

It is well known that changes in cerebrovascular or systemic physiological parameters modify outcome after cerebral ischemia. We therefore carefully monitored these parameters in randomly selected animals before, during and after ischemia. However, we did not observe any significant differences in regional cerebral blood flow (rCBF) as measured by laser Doppler flowmetry, mean arterial blood pressure (MABP), blood gases (pH, $PaCO_2$ and PaO_2) or core (rectal) temperature between groups (Table 1). Together, it seems unlikely that changes in brain perfusion of systemic physiological parameters contributed to the differences seen in stroke outcome.

DISCUSSION

This study provides genetic evidence that reduced but not abolished levels of Dnmt1 in postmitotic neurons protects tissue within ischemic territory after experimental stroke in mice. The major finding is a significant protection observed in animals lacking one allele of *Dnmt1* in postnatal neurons (1lox/+), but not in animals with a conditional deletion (1lox/c). Neurological deficits were not significantly reduced in heterozygous mice along with the infarct sparing although there was a trend to improved scores. There were no group differences in genetic background or differences in physiological or cerebrovascular parameters to explain these results.

Our findings corroborate our previous report using *Dnmt1* constitutional mutants [8]. We previously demonstrated that DNA methylation activity increases following brain ischemia in wildtype mice after reperfusion but not in mutant animals lacking one allele of *Dnmt1* (i.e. $Dnmt1^{s/+}$ mice) [8]. $Dnmt1^{s/+}$ mice were resistant to
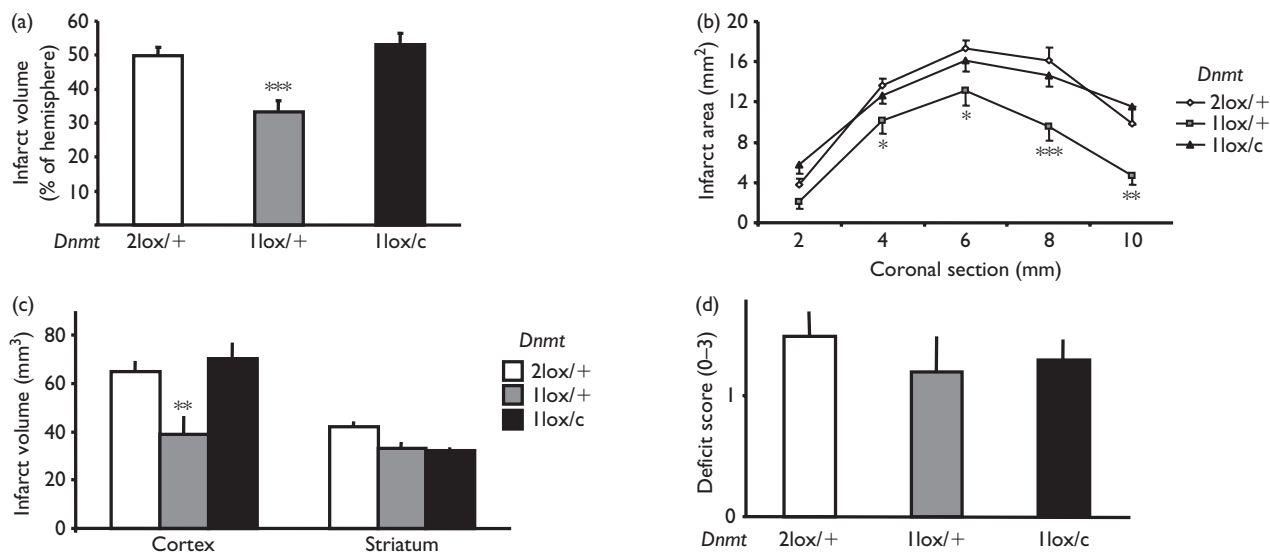
Fig. 1. Effects of 1 h middle cerebral artery occlusion and 23 h of reperfusion in *Dnmt1*[2lox/+] (wildtype), *Dnmt1*[1lox/+] (heterozygotes) and *Dnmt1*[1lox/c] (conditional mutants) mice. (**a**) Brain infarct volume was significantly smaller in mice lacking one allele of *Dnmt1* (1lox/+) in postmitotic neurons compared to wildtype littermate controls (2lox/+). Cerebral infarct volume was determined quantitatively and expressed as percent of total hemisphere volume as described [8,12]. Conditional *Dnmt1* mutant mice (1lox/c) were not different from controls and infarcts were significantly bigger than in heterozygote animals. (**b**) Brain infarct areas were significantly smaller in four of the five standardized 2 mm coronal sections in *Dnmt1*[1lox/+] (gray square) than in *Dnmt1*[2lox/+] (white diamond) and *Dnmt1*[1lox/c] (black triangle) mice. (**c**) Infarct sparing in *Dnmt1* 1lox/+ mice was prominent in the cortex while striatal tissue injury was similar between groups. (**d**) Neurological sensory motor deficits were not signficantly different between groups at 24 h, although there was a trend for improved scores in the *Dnmt1* [1lox/+] mice. Deficits were evaluated by a naïve observer using an established rating system ranging from 0 (no deficit) to 3 (severe). Bars indicate mean ± s.e. (n = 10–13 animals/group). $*p < 0.05$; $**p < 0.01$; $***p < 0.001$ compared to *Dnmt1*[2lox/+] (wildtype) mice. ANOVA and Tukey's test (a–c) or ANOVA on ranks (d).

Table 1. Physiological variables before, during and after cerebral ischemia/reperfusion.

Parameter	*Dnmt1*[2lox/+]	*Dnmt1*[1lox/+]	*Dnmt1*[1lox/c]
MABP (mmHg)			
baseline	89 ± 3	87 ± 2	93 ± 5
during	88 ± 3	85 ± 3	96 ± 8
after	96 ± 3	93 ± 4	100 ± 3
pH			
baseline	7.36 ± 0.01	7.32 ± 0.01	7.32 ± 0.03
after	7.31 ± 0.01	7.30 ± 0.03	7.29 ± 0.02
PaO₂ (mmHg)			
baseline	167 ± 7	164 ± 5	167 ± 4
after	132 ± 10	157 ± 13	139 ± 10
PaCO₂ (mmHg)			
baseline	38 ± 2	41 ± 2	40 ± 2
after	45 ± 2	40 ± 2	46 ± 3
rCBF (%)			
before	100 ± 0	100 ± 0	100 ± 0
during	17 ± 1	17 ± 2	14 ± 2
after	85 ± 5	93 ± 4	82 ± 9
weight (g)	22.2 ± 0.9	21.8 ± 0.9	22.7 ± 0.9
CT (°C)			
during	36.8 ± 0.1	36.9 ± 0.1	36.8 ± 0.1

Animals were subjected to 1 h filamentous middle cerebral artery occlusion followed by reperfusion. MABP (mean arterial blood pressure) and rCBF (regional cerebral blood flow) were measured at baseline, during ischemia, and following 30 min after reperfusion [8,12]. Fifty microliters of blood were withdrawn twice, before ischemia and directly before reperfusion for blood gas determination. Animals were weighed before the onset of the experiment (body weight in g). Core temperature (CT) was controlled and recorded by means of a feedback temperature control unit. There were no statistically significant differences between groups (n = 8–9 animals each group). Values are mean ± s.e.

ischemic damage, suggesting that increased DNA methylation is associated with augmented brain injury after MCA occlusion. From these experiments, however, it was not clear if the observed differences were due to effects of *Dnmt1* deficiency during development or altered tissue responses in the adult brain. Moreover, we could not differentiate between neuronal *vs* non-neuronal effects. This present study now unequivocally demonstrates that reduced Dnmt1 levels in postmitotic CNS neurons of the postnatal brain confer resistance to experimental stroke injury.

Experiments using constitutional deletions were limited by the fact that homozygous mutants die during embryogenesis. Using the CamK-Cre-driven loop-out approach we were now able to investigate stroke susceptibility in mice with no measurable Dnmt1 expression in neurons. Surprisingly, these mice were not protected from cerebral ischemia/reperfusion. Instead, they had the same infarct size as wildtype controls and had significantly bigger lesions than heterozygous litters. This observation is reminiscent of the results obtained with p53 knockout and p53 heterozygous mice: although the absence of p53 was protective in a mouse model of cerebral ischemia, greater protection was afforded by reduced expression of p53 [14]. Of note, conditional *Dnmt1* mutants appeared phenotypically normal and gross and microscopic anatomy revealed no overt anomalies [9,10]. Moreover, conditional mutants were recovered at the expected Mendelian ratio, indicating that Dnmt1 deficiency in postmitotic DNA neurons did not affect animal viability [9,10]. Hence, our results indicate

that abolished (as opposed to reduced) levels of Dnmt1 are not advantageous in situations of acute tissue injury such as after cerebral ischemia.

Recent studies suggested that methylated cytosine and the proteins that bind it are essential for both proper CNS survival and function [15]. We had originally proposed two (mutually non-exclusive) mechanisms how DNA methylation influences ischemia outcome and why reduced levels of Dnmt1 confer neuroprotection after stroke: (1) DNA methylation is an epigenetic mechanism to differentially influence gene expression patterns. Hence, neuroprotection may relate to ischemia-induced changes in gene expression mediated by DNA methylation. (2) DNA methyltransferase could enhance mutagenesis by facilitating C-T and C-U transitions directly [8,16,17]. Thus, a direct genetic mechanism by which Dnmt1 contributes to ischemic damage seems feasible. In the light of the somewhat counterintuitive results reported in this study it seems unlikely that Dnmt1-induced mutagenesis is the main mechanism: if so, we would have expected a linear correlation between Dnmt1 levels and brain damage, and the conditional mutant mice would have been expected to have smaller infarcts than their heterozygous littermates. Therefore, our results support the notion that neuroprotection may relate to ischemia-induced changes in gene expression mediated by DNA methylation. As an epigenetic factor, DNA methylation patterns may be subject to active regulation in response to cerebral ischemia and in return modify gene expression. According to a 'good gene *vs* bad gene' theory, it is feasible that animals with reduced Dnmt1 levels up-regulate protective genes or repress deleterious genes leading to neuroprotection and that this is not possible in conditional mutants. There are a number

of transgenic mice with various levels of Dnmt1 expression in the brain available that could be used to perform gene dose–response experiments [4,5,9,11,18].

CONCLUSION
We demonstrate that reduced levels, but not an absence, of *Dnmt1* in postmitotic CNS neurons protects the brain from cerebral ischemia/reperfusion. These observations underscore the complex role of Dnmt1 to mechanisms of injury and repair in the postnatal brain.

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